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Insights Into Sleep Homeostasis From A Drosophila Genetic Screen For Sleep Rebound Mutants

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Abstract

Sleep rebound – the increase in sleep that follows sleep deprivation (SD) – is a hallmark of homeostatic sleep regulation that is conserved across the animal kingdom. However, both the mechanisms that underlie sleep rebound and its relationship to other forms of homeostatic sleep regulation remain unclear. To identify mechanisms important for sleep rebound, we developed a novel method of inducing SD in Drosophila by thermogenetically activating wake-promoting neurons. We then used this method to conduct a large-scale genetic screen to identify Drosophila mutants with reduced sleep rebound. In Chapter 1, we discuss the use of Drosophila melanogaster as a model organism in sleep research. In Chapter 2, we discuss results of the genetic screen, where we find that sleep rebound and habitual sleep amount are controlled by separate genetic factors. In Chapter 3, we present data suggesting that mutants with reduced sleep rebound experience a milder wake-promoting stimulus during the sleep deprivation period compared to control flies, and that this difference in the strength of the wake-promoting stimulus is likely responsible for the reduced rebound phenotype. In Chapter 4, we discuss the implications of these data, and future directions to explore a model where homeostatic plasticity in the neural circuit used to produce sleep loss is responsible for subsequent rebound. These findings have important implications for our understanding of sleep and provide a model for homeostatic sleep regulation that could apply to mammalian systems.

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ABSTRACT

INSIGHTS INTO SLEEP HOMEOSTASIS FROM A *DROSOPHILA* GENETIC SCREEN

FOR SLEEP REBOUND MUTANTS

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Sleep rebound – the increase in sleep that follows sleep deprivation (SD) – is a hallmark of homeostatic sleep regulation that is conserved across the animal kingdom. However, both the mechanisms that underlie sleep rebound and its relationship to other forms of homeostatic sleep regulation remain unclear. To identify mechanisms important for sleep rebound, we developed a novel method of inducing SD in *Drosophila* by thermogenetically activating wake-promoting neurons. We then used this method to conduct a large-scale genetic screen to identify *Drosophila* mutants with reduced sleep rebound. In Chapter 1, we discuss the use of *Drosophila melanogaster* as a model organism in sleep research. In Chapter 2, we discuss results of the genetic screen, where we find that sleep rebound and habitual sleep amount are controlled by separate genetic factors. In Chapter 3, we present data suggesting that mutants with reduced sleep rebound experience a milder wake-promoting stimulus during the sleep deprivation period compared to control flies, and that this difference in the strength of the wake-promoting stimulus is likely responsible for the reduced rebound phenotype. In Chapter 4, we discuss the implications of these data, and future directions to explore a model where homeostatic plasticity in the neural circuit used to produce sleep loss is responsible for subsequent rebound. These findings have important implications for our understanding of sleep and provide a model for homeostatic sleep regulation that could apply to mammalian systems.

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This chapter is an excerpt from "Circadian rhythms and sleep in *Drosophila melanogaster*" published in *Genetics* (2017) DOI:10.1534/genetics.115.185157

Introduction

Sleep research in *Drosophila melanogaster* originated with two studies published in at the turn of the millennium (Hendricks *et al.* 2000; Shaw *et al.* 2000). In these studies, it was found that *Drosophila* periodically enter a state of quiescence that meets a series of criteria for sleep: (1) This quiescent state is characterized by an increased arousal threshold (decreased responsiveness to sensory stimuli), but (2) it can be distinguished from coma or anesthesia by its rapid reversibility with a stimulus that is sufficiently strong. (3) The timing of sleep is regulated by the circadian clock, although these two processes can also be separated; flies with mutations in the core clock genes have fragmented sleep across the day, but can have normal overall sleep amounts (Hendricks *et al.* 2003) (Figure 1-1), and flies with mutations that result in very low total sleep amounts can still show robust circadian activity rhythms. (4) Sleep is also homeostatically regulated, such that when flies are deprived of sleep using a mechanical stimulus, they compensate with longer and deeper sleep the following day. This suggests that sleep serves an important restorative function rather than simply reflecting ecologically advantageous periods of inactivity. (5) Flies experience broad changes in neuronal activity during sleep. Although rhythmic neuronal activity, like that observed with the EEG in mammals, has not been observed in flies, local field potential recordings of the protocerebral area and imaging with the optical calcium indicator GCaMP in the mushroom body show that sleep is a state of reduced neuronal activity and blunted neuronal responses to sensory stimuli (Nitz *et al.* 2002; Bushey *et al.* 2015). Importantly, many genetic and molecular regulators of sleep are conserved across species (Crocker and Sehgal 2010). Thus, sleep in flies closely resembles sleep in other organisms, and

researchers can take advantage of the benefits of this small, genetically tractable model organism to advance our understanding of the molecular neuroscience of sleep.

At the center of much sleep research is the enigmatic question: what is the function of sleep? We know that in flies as well as in mammals, important brain processes like learning and memory suffer when animals are sleep deprived, and can be recovered by allowing sleep to occur. However we do not yet know what, at a molecular level, is being depleted and restored. A related line of thought presumes that if we can understand the regulatory factors that underlie the sleep homeostasis, this will lead to a better understanding of sleep function. Gene expression studies have revealed interesting molecular signatures of sleep across the animal kingdom (Mackiewicz *et al.* 2009), and this has led to a number of interesting hypotheses about sleep function: that it is a time for particular synaptic plasticity processes (Tononi and Cirelli 2006), or specific metabolic activities (Mackiewicz *et al.* 2007), but evidence supporting these hypotheses is mixed (Scharf *et al.* 2008; Tononi and Cirelli 2014; Frank and Cantera 2014). An additional physiological correlate of sleep in mammals is greater influx of cerebral spinal fluid into the brain, which may also have a functional role, but has not been directly connected to behavior (Xie *et al.* 2013). Likewise, research in mammalian systems has uncovered at least some of the relevant neural circuitry for sleep regulation, and a flip-flop switch model for how sleep and wake states are stabilized (Saper *et al.* 2010; Weber and Dan 2016), but has not revealed satisfying mechanisms to explain what forces cause this switch to flip.

Small model organisms have great potential to reveal single genes and molecules with large impacts on sleep regulation or function, potentially providing answers to these big questions. However, work in model organisms over the past 16 years has also revealed the complexity of even evolutionarily early sleep states. Circadian and homeostatic regulation of sleep were important for establishing similarities between *Drosophila* sleep and sleep in mammals, but in addition to regulation by the circadian clock and homeostatic system, sleep in *Drosophila* can be modulated by diverse environmental factors (Zimmerman *et al.* 2012), such as social experience (Ganguly-Fitzgerald *et al.* 2006; Bushey *et al.* 2011; Chi *et al.* 2014; Liu *et al.* 2015; Lone *et al.* 2016), mating (Isaac *et al.* 2009), light (Shang *et al.* 2008), temperature (Parisky *et al.* 2016), feeding (Keene *et al.* 2010; Thimgan *et al.* 2010), age (Koh *et al.* 2006; Seugnet *et al.* 2011a; Metaxakis *et al.* 2014; Kayser *et al.* 2014), infection (Kuo *et al.* 2010; Kuo and Williams 2014), and stress (Lenz *et al.* 2015). Some of these environmental factors act on the circadian and homeostatic circuitry, but many of these environmental modulators also employ independent mechanisms that do not seem to interfere with circadian timekeeping, sleep amount when animals are undisturbed, or the homeostatic response to sleep loss. In *C. elegans*, two different sleep-like states have been described that meet nearly all the criteria above, but instead of regulation by the circadian clock, these sleep states are induced by either the molting phase of worm development or by stress (Raizen *et al.* 2008; Hill *et al.* 2014). Thus, complex regulation of sleep by diverse environmental factors is likely a general principal of sleep that can be extracted from evolutionarily primitive organisms like insects and nematodes. The picture that emerges from this work, then, is not of a uniform state with simple regulatory mechanisms, but rather of a state

that is subject to regulation by a variety of external and internal forces, which may serve different molecular functions in different neuronal or environmental contexts.

Measuring Sleep

Based on initial studies of arousal threshold, sleep in *Drosophila* is commonly defined as a period of inactivity lasting five minutes or longer (Shaw *et al.* 2000; Huber *et al.* 2004; Andretic and Shaw 2005). Sleep is typically monitored through the same *Drosophila* Activity Monitoring System (DAMS) used to analyze circadian behavior. This system relies on an active fly crossing the center of the locomotor tube to break the infrared beam passed across the middle, but this system is generally sufficient to differentiate sleep from activity in young, healthy flies, where activity levels are high enough that it is unlikely that a 5-minute or greater period of inactivity would be recorded by chance.

In old or sick flies with reduced overall activity, it may be useful to use a more sensitive method of evaluating sleep behavior. There is also the possibility that extended feeding behavior, in which a fly would dwell at the end of the tube with food and fail to cross the center beam, could be misconstrued as sleep (Cavanaugh *et al.* 2016). There are two alternatives to traditional single-beam DAMS monitors that can be used to address these concerns. Multi-beam DAMS monitors, where 17 infrared beams along the length of a locomotor tube are used to monitor activity, provide a similar environment to the traditional locomotor tube set up but offer increased sensitivity (Garbe *et al.* 2015). Video monitoring systems have also been set up to monitor the activity of individual flies (Zimmerman *et al.* 2008; Gilestro 2012; Donelson *et al.* 2012; Faville *et al.* 2015; Garbe *et al.* 2015). Video monitoring systems, while potentially offering increased sensitivity,

also present a difficulty in that no standard for the sensitivity to motion for these systems has been agreed upon. A very sensitive system may detect leg twitches or imaging artifacts during sleep and inappropriately read these as activity. Video monitoring could also introduce another potential confounding factor if it uses small arenas instead of the typical locomotor tube, as this can result in differences in behavior (Garbe *et al.* 2015). Thus, while different results can sometimes be observed between video systems and traditional DAMS monitors, these results should be interpreted with caution.

When observing a fly with reduced or elevated overall levels of sleep, it can be conceptually useful to determine how sleep bout architecture is changed (Andretic and Shaw 2005). For example, short-sleeping mutants may initiate fewer bouts of sleep, or may be unable to maintain sleep over long bouts, which implies different mechanisms of action for these genes. Most software used for automated analysis of sleep behavior allows for study of sleep bout architecture in addition to total sleep time.

Sleep depth is an additional dimension of sleep that DAMS monitoring alone does not detect, although automated systems to probe sleep depth have been developed (Faville *et al.* 2015). While initial characterizations of sleep depth suggested that sensory unresponsiveness plateaus after five minutes of inactivity, subsequent studies have demonstrated that sleep depth varies predictably over longer bouts of sleep as well. Troughs in arousability have been observed after fifteen minutes and thirty minutes of sleep, and protocerebral local field potential recordings show variation in neuronal activity based on length of sleep bouts, in some ways resembling the changes in sleep depth ("sleep stages") that occur during bouts of sleep in mammals (van Alphen *et al.*

2013). Depth of sleep also differs between day and night, such that daytime "siesta" sleep in flies is generally a lighter sleep state. Increased sleep depth is also a component of the homeostatic response to sleep deprivation (Huber *et al.* 2004; van Alphen *et al.* 2013; Dubowy *et al.* 2016), and mutations can affect sleep depth in ways that would not be predicted by changes in sleep amount (Faville *et al.* 2015).

Genetic Tools for Sleep Research

Sleep research in *Drosophila*, like a lot of molecular neuroscience in this model organism, has drawn heavily on both the study of mutations that lead to aberrant sleep behavior as well as the use of a genetic toolkit for manipulating neuronal activity. One successful strategy for identifying novel regulators of sleep is to conduct forward genetic screens for mutants with very extreme phenotypes. Another strategy is manipulation of different neuroanatomic loci, labeled by Gal4 drivers, by activating or suppressing neuronal firing. Researchers can use a variety genetic tools to manipulate neuronal activity. The bacterial sodium channel NaChBac (Luan *et al.* 2006; Nitabach *et al.* 2006) and the potassium channel Kir2.1 (Baines *et al.* 2001) can be driven either throughout fly development or in a time-restricted manner using inducible binary expression systems to activate or silence cells, respectively. Thermogenetic tools, such as the heat-activated depolarizing channel TrpA1 (Hamada *et al.* 2008; Parisky *et al.* 2008) or the temperature-sensitive dominant negative allele of *shibire* used to block synaptic transmission (Kitamoto 2001), as well as optogenetic tools, such as the light-activated depolarizing CsChrimson channel (Klapoetke *et al.* 2014) are also frequently used for conditional manipulation of neurons.

In many cases, gene- and circuit-based approaches intersect. Many, though not all, genes that regulate sleep have been shown to function in specific neuroanatomic loci, in some cases identifying novel sleep regulating areas of the fly brain. Important advances have also come from studying interactions between genes that regulate sleep, as well as studying genes that produce sleep phenotypes and have unknown or unappreciated roles in controlling neuronal activity. Studying sleep in *Drosophila* then not only leads to insight into sleep-regulatory mechanisms that may extend to mammals, but also identifies novel regulators of neuronal function and provides new insight into brain signaling and metabolism. In this review, we present a thorough discussion of the genetics and neuroanatomy of sleep, with an emphasis on how sleep regulating genes act in the context of sleep-regulating brain regions and how different sleep regulating genes and brain areas interact with each other.

Sleep regulation through global modulation of neuronal activity

The *Shaker* potassium channel (Cirelli *et al.* 2005; Bushey *et al.* 2007) and its modulator *sleepless* (Koh *et al.* 2008) were two early hits with extreme short-sleeping phenotypes from large-scale genetic screens. Both genes are expressed throughout the fly brain (Wu *et al.* 2009), and neither of these phenotypes has been fully mapped to specific neuroanatomic loci, suggesting that they exert widespread effects on brain activity or metabolism that feed back onto sleep regulation. *Shaker* is a voltage-gated potassium channel involved in membrane repolarization. *Sleepless* is a Ly6 neurotoxin-like molecule that, in the years since its discovery as a sleep regulator, has been found to promote *Shaker* expression and activity and inhibit nicotinic acetylcholine (nAChR)

function, such that loss of *sleepless* might lead to increased neuronal activity through multiple mechanisms (Wu *et al.* 2009; Shi *et al.* 2014; Wu *et al.* 2014). The molecular functions of these genes therefore suggest a mechanism of sleep regulation where wakefulness is produced by broadly increasing neuronal excitability. Indeed, broadly inhibiting cholinergic transmission partially suppresses both the *Shaker* and *sleepless* phenotypes, and RNAi knockdown of the nAChRα3 subunit suppresses the *sleepless* phenotype (Wu *et al.* 2014). However, recent work has revealed a more complicated role for these genes. Although it has typically been assumed that the *Shaker* phenotype results from increased neuronal activity of wake-promoting cells, a recent study found that knocking down *Shaker* in sleep-promoting cells actually lengthens the inter-spike interval and reduces neuronal activity in these populations to favor wake (Pimentel *et al.* 2016). Another study found that, in contrast with the generally wake-promoting effects of cholinergic neurotransmission in the fly brain (Wu *et al.* 2014; Seidner *et al.* 2015), a specific nAChR subunit, *redeye*, is strongly sleep-promoting (Shi *et al.* 2014). Genetic evidence suggests that *sleepless* also interacts with the *redeye* subunit, in this case acting as a wake-promoting rather than sleep-promoting molecule, consistent with *sleepless* inhibiting nAChRs regardless of subunit composition. Recent studies of *sleepless* have also suggested that it in part regulates sleep by non-cell autonomously promoting metabolism of GABA in glia, perhaps also through its effect on neural activity (Chen *et al.* 2014; Maguire *et al.* 2015). *Shaker* and *sleepless* thus both seem to interact in a non-straightforward way with sleep-regulatory genes and cells in the nervous system, and work with *sleepless* suggests a potential connection between neuronal activity and metabolism of neurotransmitters, although the details of this connection remain unclear.

The Mushroom Body

The mushroom body is the center of olfactory memory in the fly brain and as a result of years of intense research, there is detailed anatomic and functional data available for mushroom body circuits (Guven-Ozkan and Davis 2014; Owald and Waddell 2015). The mushroom body consists primarily of ~2000 Kenyon cells, most of which receives input from an average of 6 stochastically connected projection neurons, with each projection neuron encoding input from a single type of odorant receptor neuron. Each Kenyon cell projects axons to a subset of lobes of the mushroom body, forming three classes: those that project to the α and β lobes, the α' and β' lobes, or the γ lobe. Within each lobe there exist several compartments, defined by the dendrites of different mushroom body output neurons (MBONs) and axonal projections of different dopaminergic neurons, which respond to aversive or appetitive unconditioned stimuli like electric shock or sugar. At least some MBONs have an inherent valence, which is correlated with neurotransmitter expression; flies will act to avoid optogenetic activation of aversive glutamtergic MBONs, and act to prolong activation of attractive MBONs, which can be cholinergic or GABAergic (Aso *et al.* 2014b). A simple model of learning and memory in the mushroom body posits that within a mushroom body compartment, the strength of the synapses between Kenyon cells, which encode odor, and MBONs, which encode valence, is modulated by dopaminergic neurons in response to pairing of an odor with an aversive or appetitive stimulus (Owald and Waddell 2015). MBONs project to largely uncharacterized protocerebral areas of the fly brain (Aso *et al.* 2014a). In addition to the neurons described above, mushroom bodies also receive octopaminergic input and are innervated by the dorsal paired medial (DPM), anterior paired lateral (APL), and dorsal

anterior lateral (DAL) pairs of neurons (Guven-Ozkan and Davis 2014). These neurons may serve functions in memory consolidation or in fine-tuning olfactory coding.

The mushroom body was also the first neuroanatomic structure identified as a regulator of sleep in *Drosophila* (Joiner *et al.* 2006; Pitman *et al.* 2006). Conditional approaches were used to block synaptic transmission, perturb PKA signaling, or manipulate excitability of mushroom body neurons primarily using the relatively broad Gal4 drivers that were available at the time, but also taking advantage of methods that could target the mushroom body specifically. These approaches suggested that the mushroom body contains both sleep-promoting and wake-promoting cells: for example, flies slept less when hydroxyurea was used to ablate α/β and α'/β' mushroom body lobes, suggesting a sleep-promoting role for these cells, but slept more when a relatively restricted mushroom body GeneSwitch line was used to silence specific cells in adulthood.

Later work using more restricted split-Gal4 lines identified specific mushroom body circuits that underlie both wake- and sleep-promoting effects (Aso *et al.* 2014b; Sitaraman *et al.* 2015a). Several MBONs are capable of regulating sleep behavior, and interestingly these same MBONs are also necessary for certain types of learning and memory (Aso *et al.* 2014b). The sleep- and wake-promoting characteristics of MBONs seem to correlate with their aversive or appetitive nature, such that the two identified wake-promoting groups of MBONs are aversive and glutamatergic, whereas two identified sleep-promoting MBONs are appetitive and cholinergic or GABAergic; an unusual MBON with dendritic projections in the calyx and no identified neurotransmitter or valence is also sleep-promoting (Aso *et al.* 2014b). Wake- and sleep-promoting

characteristics of different Kenyon cell populations then seem to reflect which of the sleep-controlling MBONs the Kenyon cells in question target most prominently (Sitaraman *et al.* 2015a). For example, neural epistasis experiments suggest that the wake-promoting glutamatergic MBONs (γ5β'2a/ β'2mp'/ β'2mp_bilateral) are downstream of a wake-promoting α'β' KC driver and a broad wake-promoting KC driver that encompasses γ-dorsal, γ-main, and α/β KCs (γ-dorsal KCs are sleep-promoting on their own, but γ-main KCs are wake-promoting). On the other hand, blocking the sleep-promoting cholinergic γ2α'1 MBONs makes the wake-promoting effects of the broad KC driver that encompasses γ-dorsal, γ-main, and α/β KCs even stronger, suggesting that γ2α'1 MBONs receive sleep-promoting input from these cells, even if the net effect of the KC driver conferred by other downstream MBONs is wake-promoting. Likewise, the DPM neurons, which are proposed to provide inhibitory input to α'β' KCs via GABA and/or serotonin, are strongly sleep-promoting when activated, consistent with an overall wake-promoting effect of α'β' KCs (Haynes *et al.* 2015). Loss of the d5-HT1 serotonin receptor in mushroom bodies also produces a weak short-sleep phenotype, which can be rescued with expression driven by the MB-GeneSwitch driver (Yuan *et al.* 2006). This finding is consistent with serotonin released from DPMs acting to inhibit wake-promoting α'β' KCs.

Dopamine and the Dorsal Fan-Shaped Body

Perhaps the strongest parallel between mammalian and *Drosophila* sleep regulation identified so far is the strong wake-promoting effects of the monoamine neurotransmitters dopamine and octopamine (the insect homolog of norepinephrine,

discussed in the next section). *fumin*, one of the first short-sleeping mutants identified, is a mutation in a dopamine transporter that presumably results in elevated dopamine levels throughout the fly brain (Kume *et al.* 2005) and dopaminergic neurons are strongly wake-promoting when activated (Shang *et al.* 2011; Liu *et al.* 2012). Conversely, mutants deficient for the CNS-specific isoform of the tyrosine hydroxylase (TH), the rate-limiting enzyme for dopamine synthesis, have increased sleep throughout the day (Riemensperger *et al.* 2011).

One site of dopaminergic action is the central complex, an area of the brain that has been hypothesized to serve a basal ganglia-like function in action selection based in part on the input it receives from protocerebral areas and its functional role in motor output (Strausfeld and Hirth 2013). Thermogenetic activation of the ExFl2 neurons in the dorsal fan-shaped body, a region of the central complex, is strongly sleep-promoting (Donlea *et al.* 2011). Sleep deprivation changes the electrophysiologic properties of these neurons to favor activity, suggesting they may play a role in output of homeostatic sleep signals (Donlea *et al.* 2014). Dopamine provides a wake-promoting stimulus by silencing these neurons, although there is some disagreement regarding the relevant cluster of dopaminergic neurons as well as the relevant D1-like dopamine receptor. A MARCM approach to target single dopaminergic neurons indicated that individual PPM3s with projections to the fan-shaped body exert small but significant effects on sleep behavior, while a separate study comparing expression of wake-promoting and non-wake-promoting Gal4 drivers suggested that PPL1s with projections to the fan-shaped body provide wake-promoting input (Ueno *et al.* 2012; Liu *et al.* 2012). It is possible that both groups of cells provide wake-promoting dopaminergic input to this brain area. Likewise,

it was initially thought that Dop1R1 was the relevant receptor for wake-promoting

dopaminergic signaling in the brain. Dop1R1 mutations suppress the *fumin* phenotype

and unlike wild-type flies, Dop1R1 mutants do not experience severe sleep reduction

when fed L-DOPA (Ueno *et al.* 2012; Liu *et al.* 2012). These effects can be rescued with

Dop1R1 expression driven by the relatively specific fan-shaped body driver 104y-Gal4.

However, more recent work shows that RNAi knockdown of the related receptor Dop1R2

in the dorsal fan-shaped body ExFl2 neurons is sufficient to prevent both short-term

hyperpolarization and longer-term silencing of these cells by dopamine, and this

manipulation also produces a long-sleep phenotype (Pimentel *et al.* 2016).

Dopaminergic neurons with projections to the mushroom body also have wake-promoting

effects (Sitaraman *et al.* 2015b; Nall *et al.* 2016). Neurons of the PAM cluster, as well as

a subset of neurons of the PPL1 cluster distinct from those that project to the fan-shaped

body, project to specific compartments of the mushroom body (MB). Recent work has

suggested that MB-PAM neurons and MB-PPL1 neurons can be wake-promoting when

thermogenetically activated. The wake-promoting effects of caffeine are also mediated by

the PAM cluster of neurons (Nall *et al.* 2016). However, although Split-Gal4 drivers have

been used to elegantly identify specific mushroom body circuits that control sleep, the

PAM and PPL1 neurons that promote wake do not seem to neatly correspond to these

circuits (Sitaraman *et al.* 2015b). It is possible that diffusion of dopamine or functional

interconnectivity between dopaminergic neurons (Cohn *et al.* 2015) contributes to these

results.

Genetic knock-outs and experiments silencing dopaminergic neurons show that endogenous dopamine plays an important role in daily sleep regulation; however it is interesting that increases in global dopamine levels can be compensated with loss of the Dop1R1 receptor to achieve approximately normal amounts of daily sleep (Ueno *et al.* 2012). Thermogenetic activation of dopaminergic neurons produces a sleep rebound once activation is stopped, suggesting that these wake-promoting neurons are upstream of neuronal machinery capable of producing homeostatic responses to extended wakefulness (Seidner *et al.* 2015; Dubowy *et al.* 2016). Alterations in dopamine signaling are also implicated in sleep regulation by developmental or environmental cues; the increased sleep amounts that young flies experience have been attributed to decreased dopaminergic input to the dFSB, and dopamine has also been proposed to play a role in the adaptation of sleep amount to changing social environments (Ganguly-Fitzgerald *et al.* 2006; Kayser *et al.* 2014). In addition to inhibitory, wake-promoting input from dopamine, the dFSB may also receive input from unidentified sleep-promoting neurons labeled by the 201y-Gal4 driver (Cavanaugh *et al.* 2016). Thus, the dorsal fan-shaped body is well-positioned to act as an integrator and output for many sleep-regulatory signals.

In addition to *fumin*, other short-sleeping mutants also appear to depend on dopamine or the fan-shaped body for their mechanisms of action. The Rho-GAP *crossveinless-c* is a sleep-promoting molecule that disrupts the physiological membrane properties of the ExFl2 neurons when mutated, resulting in reduced sleep (Donlea *et al.* 2014). The 2-pore potassium channel *Sandman* is necessary for dopamine-mediated silencing of these neurons and knockdown of this channel in these neurons also produces a short sleep

phenotype (Pimentel *et al.* 2016). The spatial requirements for the sleep-promoting

ubiquitin ligase component *Cullin-3* and its interacting BTB adaptor *insomniac* have not

been established (Stavropoulos and Young 2011), but pharmacologically blocking

dopamine synthesis blocks the short-sleeping phenotypes of these mutants, suggesting

that *Cullin 3*-mediated protein turnover and dopamine signaling may interact to regulate

sleep (Pfeiffenberger and Allada 2012).

Octopamine, the Pars Intercerebralis, and the Pars Lateralis

Octopamine, the insect homolog of norepinephrine, is another wake-promoting

monoaminergic neurotransmitter (Crocker and Sehgal 2008). Mutating the enzymes

responsible for octopamine synthesis or silencing octopaminergic neurons increases daily

sleep amount, while activating octopaminergic neurons or feeding flies octopamine

decreases sleep (Crocker and Sehgal 2008; Seidner *et al.* 2015). Although octopamine

provides input to the mushroom body, the wake-promoting effects of octopamine do not

appear to be mediated by this structure. Instead, a MARCM approach identified the

octopaminergic ASM neurons, which project to the *pars intercerebralis* (PI), as sufficient

to drive increased wake when chronically activated, and the PI insulin-like peptide (ILP)-

secreting neurons as downstream mediators of octopamine signaling (Crocker *et al.*

2010). The effect sizes observed when ASM or ILP-secreting neurons are manipulated

are somewhat smaller than those observed with manipulation of all octopaminergic

neurons, so it is possible that other neurons important for the wake-promoting effects of

octopamine have not yet been found. Unlike dopaminergic neurons, activating

octopaminergic neurons produces strong sleep loss without an apparent rebound the next

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day, suggesting that octopaminergic neurons provide a wake-promoting stimulus that is able to circumvent sleep homeostasis (Seidner *et al.* 2015). This work suggests that octopaminergic neurons may be a neural substrate for environmental factors that promote wake without any apparent homeostatic compensation.

In addition to the ILP-expressing neurons, distinct sets of neurons in the PI expressing EGFR ligands and SIFamide are also sleep-promoting (Foltenyi *et al.* 2007; Park *et al.* 2014). *rhomboid (rho)*, an enzyme necessary for the production of EGFR ligands, is expressed prominently in the PI, and manipulating its activity using Gal4 drivers with expression in the PI produces sleep when *rho* is overexpressed, and wake when *rho* is knocked down (Foltenyi *et al.* 2007). SIFamide is a sleep-promoting insect neuropeptide expressed in four PI neurons; ablation of these neurons or knockdown of the peptide with RNAi decreases sleep (Park *et al.* 2014). Both *rho* and the SIFamide receptor (*SIFaR*) are required in c767-Gal4 labeled neurons for normal sleep amounts, suggesting that SIFamide acts through inter-PI signaling, and implicating EGFR ligands as a possible output from this circuit (Foltenyi *et al.* 2007; Park *et al.* 2014). However, c767-Gal4 also drives expression outside the PI, and so a function of these molecules outside the PI cannot be excluded. EGFR signaling in clock neurons may mediate the effects of social enrichment on sleep, although it is not clear if this is functionally related to the release of EGFR ligand from the PI (Donlea *et al.* 2009).

A separate, but related neuroendocrine structure, the *pars lateralis* (de Velasco *et al.* 2007), was recently identified as a site of action for cell cycle regulators that modulate sleep in adult post-mitotic neurons. Two cell cycle regulators, *Rca1* (Regulator of Cyclin

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A) and *taranis* (*tara*, a Trip-Br family transcriptional co-regulator), were independently identified in genetic screens for short-sleeping mutants, and following the identification of *Rca1* it was found that knocking down *Cyclin A* (*CycA*) itself in neurons produces an equally strong short-sleeping phenotype (Rogulja and Young 2012; Afonso *et al.* 2015). *CycA* and *tara* genetically interact, and TARA binds to and post-transcriptionally promotes stable expression of Cyclin A in PL neurons (Afonso *et al.* 2015). Post-mitotic expression of *Cyclin A* is relatively restricted in the fly brain but includes ~14 neuroendocrine cells in the *pars lateralis*, and knocking down *tara* in this structure partially recapitulates the short sleeping phenotype of *tara* mutants. Experimentally activating and silencing these neurons supports a wake-promoting role. No mechanism has yet been identified for the involvement of these cell cycle regulators in neuronal activity or sleep, but this will be an interesting area of future research.

Clock Regulation of Sleep

The circadian clock is essential for restricting sleep to environmentally advantageous times of day. A role for the circadian clock has been established in flies in both putting flies to sleep at night once the dark period has begun and waking them up in advance of dawn (Liu *et al.* 2014; Kunst *et al.* 2014). Interestingly, these pathways seem to mechanistically diverge, suggesting that circadian regulation of sleep is not driven by continuous oscillation of a single sleep- or wake-promoting factor, but is rather driven by time-of-day specific modulation of distinct sleep- and wake-promoting mechanisms. Clock cells also have broader non-circadian roles in sleep regulation as mediators of the

effects of temperature and social enrichment on sleep (Donlea *et al.* 2009; Parisky *et al.* 2016).

One particularly well-studied mechanism of circadian sleep regulation regulates sleep around the time of lights-off and is driven by cyclic expression of a gene that regulates responsiveness to neuronal signals in a specific set of clock neurons. The large ventral lateral neurons (lLNvs) are a wake-promoting population of neurons with neuronal activity that fluctuates over the course of the day such, that firing frequency is reduced around the time of lights-off as well as later in the night in an LD cycle (Sheeba *et al.* 2008b; Cao and Nitabach 2008; Sheeba *et al.* 2008a; Parisky *et al.* 2008; Shang *et al.* 2008; Liu *et al.* 2014). Manipulations of lLNv activity produce broad effects on sleep and wake throughout the day, but effects are particularly pronounced at night, with clear effects on length to sleep onset (sleep latency) after lights-off. Genetic and pharmacological studies suggest that the silencing of these neurons during this time is mediated by GABA-A receptor *Rdl*, and indeed, broadly silencing GABAergic neurons in the brain substantially lengthens the sleep latency after lights-off in flies (Agosto *et al.* 2008; Parisky *et al.* 2008; Chung *et al.* 2009). The positive and negative arms of the molecular clock also oppositely regulate sleep latency such that *Clock* and *cyc* mutants have increased sleep latency after lights-off, while *per* and *tim* mutants have shortened sleep latency after lights-off (Liu *et al.* 2014). Thus, changes in activity in this circuit seem to drive sleep in response to time of day around the transition to darkness.

A key molecular mediator of these changes in activity was initially discovered in a genetic screen for short-sleeping mutants. Although *wide awake (wake)* mutant flies were

found to have reduced sleep across the day and night, which may be due to activity of *wake* in other neuroanatomic loci, the increased latency to sleep in *wake* mutants could be anatomically mapped to the lLNvs (Liu *et al.* 2014). Transcription of *wake* was found to cycle in the lLNvs, with increased transcription and protein levels at dusk, and WAKE physically and genetically interacts with the *Rdl* GABA-A receptor. Crucially, *wake* mutants did not display rhythms in lLNv firing frequency, and GABA-induced inhibitory currents in lLNvs were dampened. Thus, clock-driven expression of *wake* in lLNvs appears to be the key time-of-day driven regulator that induces sleep after lights-off.

A distinct mechanism in a different set of clock neurons is invoked to promote wake at the end of the night, just before lights-on. Diuretic Hormone 31 (DH31) is a wake-promoting neuropeptide expressed in the DN1 clock neurons, and manipulating its expression in these cells produces sleep phenotypes specifically from ZT21-24: knockdown of the peptide in DN1s increases sleep during this time period, while overexpression of the peptide in these cells decreases it (Kunst *et al.* 2014). Expressing a tethered PDF peptide in the DN1s, which should produce PDFR activation in these cells, also reduces sleep specifically during late night, as does pan-neuronal expression of tethered DH31. The time-specific effects of DH31 might therefore be gated both by time-specific PDF responsiveness in DN1s and by time-specific DH31 responsiveness in downstream neurons.

However, DN1s are sleep-promoting at other times of day; optogenetically or thermogenetically activating these cells increases daytime sleep, suppressing the normal "evening" peak of activity, while silencing them decreases sleep during early night (Guo

et al. 2016). The sleep-promoting effects of DN1s during the day can be blocked with RNAi targeting mGluR in "E" cells, suggesting "E" cells might also have a role in sleep regulation. The role of sleep-promoting signals from DN1s in normal daily sleep regulation remains unclear, although the authors propose that variations in activity of DN1s may explain sexually dimorphic sleep patterns and regulation of sleep by high temperature.

Metabolic Regulation of Sleep

Food availability is a potent environmental regulator of sleep in fruit flies. Starvation strongly suppresses sleep, perhaps so that flies can devote more time to foraging for food (Keene *et al.* 2010; Thimgan *et al.* 2010). Mechanisms that regulate sleep at baseline and in response to food availability have some overlap with pathways that regulate metabolic energy storage, but these pathways are ultimately separable, such that sleep phenotypes do not depend on differences in metabolic stores (Erion *et al.* 2012; Masek *et al.* 2014; Murakami *et al.* 2016). Pharmacological evidence suggests that the suppression of sleep in response to starvation can be mimicked by feeding flies a glycolysis inhibitor, but not an inhibitor of fatty acid β-oxidation, suggesting that the suppression of sleep with starvation is related to reduced metabolic mobilization of glucose, not the taste of sugars or to lipid metabolism (Murakami *et al.* 2016).

An essential molecular mediator for these effects was recently identified in the nucleotide binding protein *translin* (Murakami *et al.* 2016). *Translin* is highly upregulated upon starvation, and *translin* knockdown completely prevents starvation-induced sleep loss in flies. However, other sleep and starvation-related behaviors, such as sleep at baseline and

after sleep deprivation, preference for sucrose or yeast after starvation, and the proboscis extension reflex following starvation are completely unaffected, and there is no evidence that *translin* knockdown alters energy stores. The effects of this molecular mediator were mapped to neurons expressing the neuropeptide leucokinin. Like *translin* knockdown, silencing leucokinin neurons prevented sleep suppression in response to starvation.

Although pharmacology suggests that sleep suppression in response to starvation is related to glucose metabolism, and is mechanistically distinct from the response to mechanical sleep deprivation, which induces a homeostatic response, a separate body of work suggests that genes involved in lipid metabolism can specifically modulate the rebound response to mechanical sleep deprivation (Thimgan *et al.* 2010; 2015). However, a mechanism through which lipid metabolism modulates sleep following sleep deprivation, or neuronal substrates of this process, remain unknown, and it is still unclear whether lipid metabolic stores are directly related to these phenotypes, or whether lipid metabolism and sleep homeostasis share common pathways.

Homeostatic Response to Sleep Deprivation

Sleep homeostasis ensures that flies sleep the proper amount by recovering lost sleep after periods of extended wakefulness. Sleep homeostasis is often conceptualized as a continuous build-up of sleep need over periods of wakefulness and dissipation over periods of sleep, such that the same mechanisms should be invoked both when flies are spontaneously waking and during periods of forced wakefulness (sleep deprivation). However, recent work in *Drosophila* has called this view into question.

A disconnect between regulation of sleep following spontaneous wakefulness and sleep following sleep deprivation is supported by a number of observations. While it is true that many short-sleeping mutants have impaired sleep rebound, these phenotypes may arise from the general inability to initiate or maintain sleep in these flies, such that even high sleep pressure cannot overcome these deficits; it is also difficult to interpret rebound data from short-sleeping flies because their habitual short sleep means they have less sleep to lose. Indeed, the converse relationship does not seem to hold: a number of genetic perturbations have been identified that specifically affect sleep after sleep deprivation with little to no effect on baseline sleep, suggesting that sleep following sleep deprivation is produced by an independent mechanism (Seugnet *et al.* 2011b; Thimgan *et al.* 2015; Seidner *et al.* 2015; Dubowy *et al.* 2016; Liu *et al.* 2016).

Likewise, it seems that the nature of sleep deprivation matters for the homeostatic response. Activating different populations of wake-promoting neurons in the fly brain produces varying amounts of rebound the following day, ranging from no rebound response at all, as is seen with activation of octopaminergic neurons, to a rebound that in some cases far exceeds the amount of sleep lost (Seidner *et al.* 2015; Dubowy *et al.* 2016). Different environmental stimuli used to keep flies awake can also produce the varying effects. Particularly strikingly, one group has reported that starving flies produces equivalent amounts of sleep loss as mechanical sleep deprivation without producing any observable sleep rebound (Thimgan *et al.* 2010). It is possible that even different mechanical sleep deprivation approaches invoke different neural pathways to keep flies awake, which may explain why so few mutants with impaired sleep rebound have been validated across labs.

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Despite these challenges, there is a picture emerging about the relevant circuitry for sleep homeostasis. Groups of wake-promoting cells that do produce sleep rebound after activation include dopaminergic neurons as well as at least one restricted set of cholinergic cells, which produce a particularly strong rebound with even short periods of activation (Seidner *et al.* 2015; Dubowy *et al.* 2016). In addition, electrophysiology suggests that the sleep-promoting dorsal fan-shaped body neurons have reduced input resistance and reduced membrane time constants, suggesting greater activity following sleep deprivation (Donlea *et al.* 2014); as discussed previously, this brain area is well-positioned to act as an integrator or output for multiple sleep regulatory signals, including, it seems, the response to sleep deprivation. It has also been suggested that silencing MBON-γ2α'1 neurons can block sleep rebound, although the data do not exclude the possibility that this is due to a general wake-promoting effect of silencing MBON-γ2α'1 neurons during the early day when rebound occurs (Sitaraman *et al.* 2015a).

A recently identified element of sleep-regulatory circuitry with an apparently specific role in sleep homeostasis is the ellipsoid body R2 neurons (Liu *et al.* 2016). These neurons were initially of interest because they produce a persistent sleep-promoting signal when thermogenetically activated; while no changes in sleep are reported at the time of activation, which can be as short as 30 minutes, a dramatic rebound-like increase in sleep is observed for the next 12 hours. Structural plasticity in the R2 neurons seems to underlie the phenotype, as circuit-specific analysis of *bruchpilot* expression showed greater synapse number and size for R2 neurons after sleep deprivation, and genetic manipulations that block this plasticity partially block sleep rebound. A neuronal epistasis

experiment suggests that these cells are upstream (although not necessarily directly connected to) the dorsal fan-shaped body. The manipulations of R2 neurons that affect sleep rebound have no effect on sleep at baseline, however, again supporting the idea that regulation of the homeostatic response to sleep deprivation is mechanistically different from the regulation of baseline sleep.

Function of Sleep

Sleep affects neurobehavioral performance across the animal kingdom, and flies are no exception. Sleep has a bidirectional relationship with learning and memory; sleep deprivation in adult flies has been shown to interfere with both short and long term memory, while inducing sleep allows memories to form in contexts where an experience would ordinarily be forgotten (Ganguly-Fitzgerald *et al.* 2006; Seugnet *et al.* 2008; Donlea *et al.* 2011; Dissel *et al.* 2015; Berry *et al.* 2015). Sleep loss also has consequences for social behavior in flies; in adult flies, acute sleep loss results in impaired aggressive behavior (Kayser *et al.* 2015). There also appears to be a critical window during development where sleep loss produces long-lasting deficits in courtship behavior and short-term memory that persist into adulthood (Seugnet *et al.* 2011a; Kayser *et al.* 2014). Precisely how these deficits arise, however, remains an outstanding question in the field.

One general line of thought supposes that there are brain-wide molecular pathways that are different during sleep and wake, and perturbed by sleep loss, that underlie these behavioral changes. Indeed, molecular characterization comparing sleeping, spontaneously waking, and sleep-deprived brains have found widespread differences in

gene expression between different behavioral states (Zimmerman *et al.* 2006; Cirelli 2006; Williams *et al.* 2007). The types of changes observed appear to be conserved across organisms: broadly, genes involved in synaptic plasticity/function, cellular stress, and metabolism are affected by sleep and wake across species studied (Mackiewicz *et al.* 2009).

One hypothesis based on this data, put forth by Tononi and Cirelli, proposes that global synaptic downscaling occurs during sleep to counteract overpotentiation that might occur during wake (Tononi and Cirelli 2006). Work from these authors shows evidence that, broadly and within specific circuits of the adult fly brain, synaptic markers increase after wake or sleep deprivation and decrease following sleep, suggesting changes in the number or size of synapses (Gilestro *et al.* 2009; Bushey *et al.* 2011). Several shared regulators of learning, synaptic plasticity, and sleep have been identified, but a direct link between synaptic plasticity and either sleep regulation or neurobehavioral performance has been difficult to establish (Bushey *et al.* 2009; Bai and Sehgal 2015; Robinson *et al.* 2016). In some cases it seems that the effects of sleep and synaptic plasticity can in fact be separated; for example, in the learning-impaired mutant *dunce*, inducing sleep improves learning, even though the global changes in synaptic markers typically associated with sleep are not observed (Dissel *et al.* 2015).

Another hypothesis states that sleep is a time where metabolic functions such as macromolecule biosynthesis can be carried out in the brain in the absence of the more urgent metabolic demands of waking. This may also explain why extended sleep loss results in induction of cellular stress genes. As with learning and synaptic plasticity,

shared regulators of metabolic or cellular stress and sleep regulation or function have been identified (Shaw *et al.* 2002; Naidoo *et al.* 2007; Thimgan *et al.* 2010; 2015; Maguire *et al.* 2015), and flies increase sleep following a heat pulse that induces a cellular stress response (Lenz *et al.* 2015), but a direct link that would establish cellular metabolism as an essential function of sleep has not yet been shown.

Some progress in understanding neurobehavioral changes with sleep comes from examining specific neurotransmitter systems or circuits that are perturbed by sleep loss. In the case of learning deficits with sleep loss, performance can be restored by overexpressing Dop1R1 or pharmacologically promoting dopamine signaling (Seugnet *et al.* 2008). In the case of loss of aggression after sleep loss, feeding flies the dopamine precursor L-DOPA does not improve behavior, but instead an octopamine agonist is effective at restoring aggression (Kayser *et al.* 2015). Studying the mechanisms that allow increased sleep to promote memory have also yielded insights; in particular, recent work suggests that inducing sleep may promote the formation of an aversive olfactory memory by suppressing a dopamine-dependent "active forgetting" process that occurs when flies are awake and moving (Berry *et al.* 2015). Whether these changes in neurotransmitter pathways are downstream of global metabolic or plasticity pathways that are altered during sleep will be an interesting area of future research.

Conclusions

The study of sleep in *Drosophila* has allowed us to harness the power of forward genetics to make significant advances in the study of sleep and neuroscience more broadly. The neuroanatomy of sleep in *Drosophila*, while not comprehensive, has identified a diverse

set of neurons in the fly brain that can regulate sleep (Figure 1-2). We are also moving towards a better understanding of how these circuits interact with each other, which will enable us to build models for how sleep regulation works that can be applied to mammalian brains. The neuroanatomy and neurochemistry of sleep in *Drosophila* includes many parallels between flies and mammals. Disruptions of potassium channel function have profound effects on sleep in flies and are also linked to human sleep phenotypes (Cornelius *et al.* 2011; Allebrandt *et al.* 2011). The wake-promoting effects of catecholamines and the sleep-regulatory roles of hypothalamus-like structures are strong parallels between flies and mammals, and the direction of sleep regulation for most neurotransmitters is preserved across evolution (Crocker and Sehgal 2010). The insect mushroom body and the central complex, on the other hand, have less clear parallels to mammalian sleep-regulatory neuroanatomy, but may still share functional homology to mammalian sleep-regulatory circuits. A better understanding of protocerebral areas of the fly brain, many of which are relatively unexplored but have connections to both the mushroom body and the central complex, may also lend insights into sleep function and regulation.

An important lesson already apparent from studying *Drosophila* is that sleep regulation is orchestrated by a complex set of genes, neurons, and environmental conditions. Although there is a tendency in the field to reduce sleep regulation to a homeostatic and a circadian component, this thinking has not been sufficient to understand sleep regulation in flies, and perhaps also in other systems. Instead, there appear to be different sets of genes and cells that regulate basal sleep drive, sleep in response to environmental cues, as well as sleep in response to forced wakefulness. Likewise, the circadian component is comprised

of different cell groups and different circadian output molecules regulating sleep and wake at specific times of day, not a single oscillating signal.

Sleep also has profound affects on waking behavior in Drosophila, making flies suitable model organisms to study the function of sleep. Excitingly, we are learning more and more about how complex behaviors are orchestrated in flies, providing more power to examine specifically how sleep and wake impinges on these processes. As we enter an era where identifying more precise mechanisms for the effects of sleep on biological functions is possible, we can begin finding commonalities across different behaviors and processes influenced by sleep, and use these findings to make general statements about what sleep does to make it necessary across the animal kingdom.

Figure 1-1: *Drosophila* sleep behavior in light:dark and dark:dark cycles
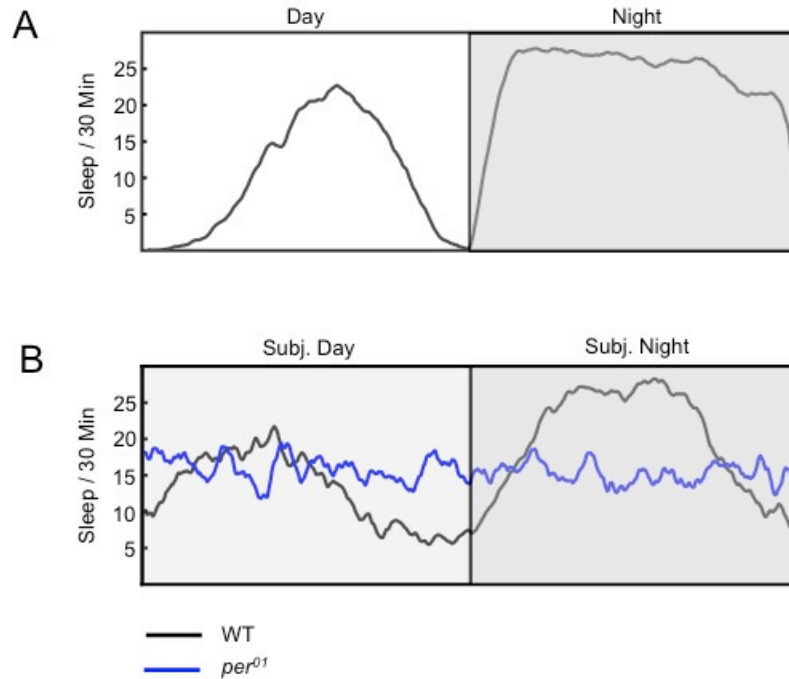
(A) Sleep behavior for a group of wild-type (WT) female flies in a 12:12 hr light:dark cycle. Flies have short bouts of siesta sleep in the middle of the day (more pronounced in males) and a relatively consolidated period of sleep at night. **(B)** Sleep behavior for WT and per^{01} male flies in constant darkness (DD). per^{01} flies, which do not display circadian rhythms of activity, spend approximately the same amount of time in sleep, but have sleep that is fragmented across the day. Data appear slightly noisier as fewer flies are represented compared to (A).

Figure 1-2: Neuroanatomy of Sleep in *Drosophila*
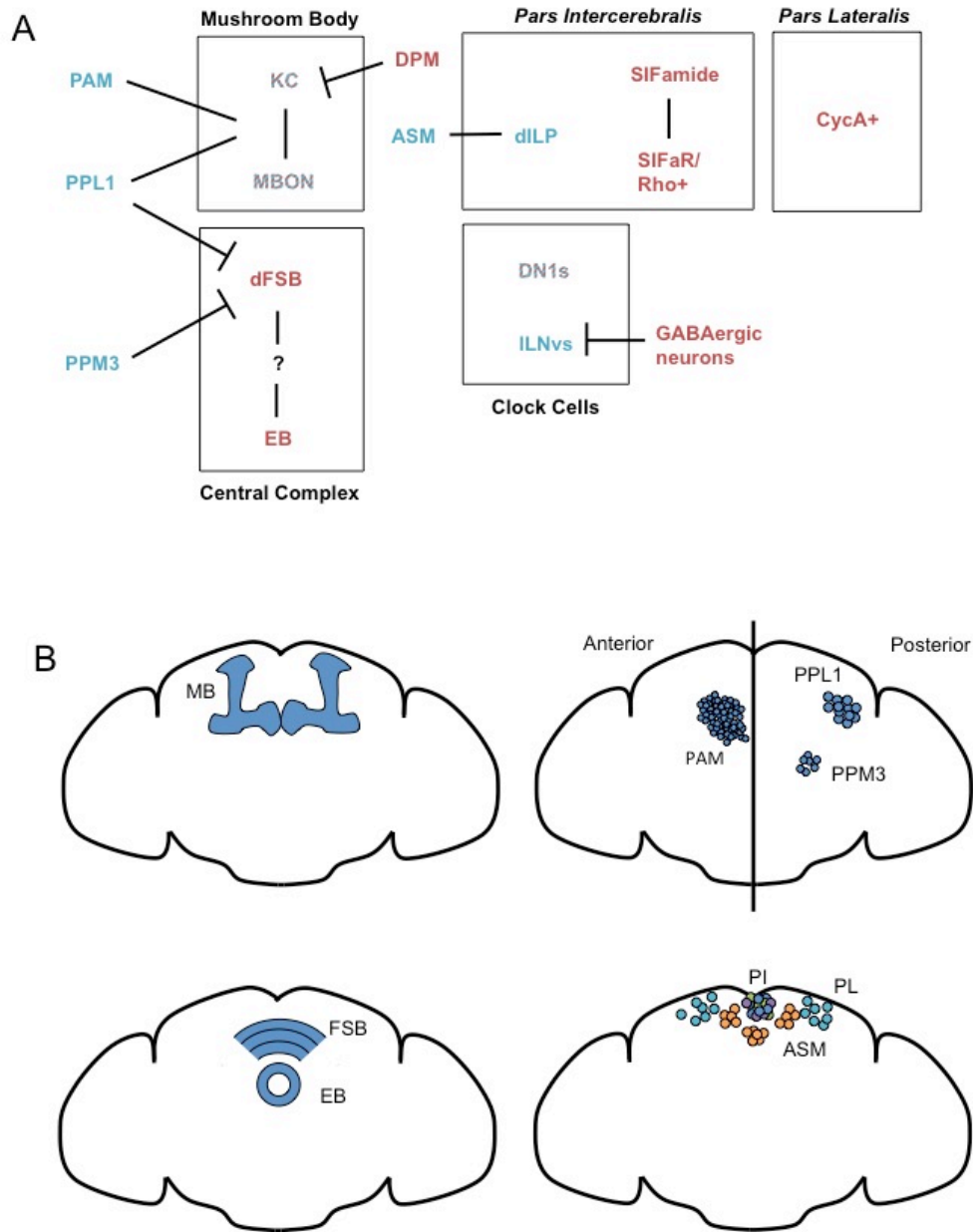
Figure 1-2: Neuroanatomy of Sleep in Drosophila melanogaster (A) Schematic of sleep promoting (red), and sleep-inhibiting (blue), neurons in the fly brain. Sleep-regulating neurons are identified by neurotransmitter, neuropeptide, or molecular marker expression, and/or neuroanatomic location. Dopaminergic neurons: PAM, protocerebral anterior lateral; PPL1, protocerebral posterior lateral; and PPM3, protocerebral posterior medial. Mushroom body neurons: KC, Kenyon cells; MBON, mushroom body output neurons. Central complex, dFSB; dorsal fan-shaped body; EB, ellipsoid body. Pars intercerebralis (PI): SIFamideR, SIFamide Receptor; Rho, rhomboid; and dILP, Drosophila insulin-like peptide. Octopaminergic neurons: ASM, anterior superior medial. Pars lateralis: CycA, CyclinA. Clock cells: DN, dorsal neurons, lLNvs, large ventral lateral neurons. (B) Location of sleep-regulating neurons in the fly brain.

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Chapter 2: Genetic Dissociation of Daily Sleep and Sleep Following Thermogenetic Sleep Deprivation in *Drosophila*

Acknowledgements

Introduction

Sleep is a fundamental biological phenomenon important for both survival and proper brain function; however, we are just beginning to identify its molecular underpinnings.[1] A physiological model of sleep regulation proposes that sleep is regulated by two independent processes: a circadian process, which regulates sleep based on time of day, and a homeostatic process, which regulates sleep based on accumulated sleep need.[2, 3] The molecules that drive the circadian process were first identified in *Drosophila* with forward genetic screens,[4, 5] and conserved mechanisms were subsequently found in mammals.[6, 7] The genes identified in these screens exhibit cycles in expression and activity over the course of the day and their cycling drives a diverse set of circadian behaviors and physiological processes.[8] However, identifying equivalent molecules that can fully explain homeostatic sleep regulation has been challenging.

Homeostatic sleep regulation is reflected both in the normal build-up of sleep pressure during spontaneous wakefulness, and in the further increase or "rebound" sleep after sleep deprivation (SD). The widely acknowledged two-process model proposed by Borbély and Daan and colleagues predicts that the same mechanisms should drive sleep pressure under both conditions.[2, 3] Indeed, electroencephalogram (EEG) slow wave activity (SWA), a widely used marker of sleep need, builds up with similar dynamics during undisturbed wake and acute SD conditions, supporting this idea.[9] However, the relevance of SWA remains unclear,[10–13] and there are conflicting accounts regarding the increase in SWA and sleep amount under conditions of chronic sleep restriction or deprivation[10,14–19] Different types of SD producing equivalent sleep loss have also been

shown to result in differential homeostatic responses in mice, as evidenced by different responses in multiple sleep latency tests despite equivalent SWA responses during recovery sleep.[20] Neurobehavioral performance after SD can also be described by the two-process model,[21] but as with sleep regulation, unexpected results have also emerged from chronic sleep restriction studies.[21–23]

Attempts to identify molecular substrates of sleep homeostasis in mammals have not yet provided a mechanistic account of sleep drive.[24] Adenosine, as well as its upstream activators prostaglandin D and nitrous oxide, growth hormone-releasing factor, tumor necrosis factor, and interleukin-1β, meet the minimal criteria of a sleep homeostasis substrate: these molecules increase during SD, and are sufficient to drive sleep when infused into the brains of mammals.[25,26] However, the effects of knocking down the receptors for these molecules or pharmacologically inhibiting these pathways tend to be either subtle or restricted to specific aspects of sleep homeostasis, i.e., EEG parameters or sleep following SD, suggesting that none of these alone can account for the entire homeostatic component of the two-process model.[27–35] This raises the possibility that there exist multiple mechanisms of homeostatic sleep regulation,[36] which account for different aspects of the proposed homeostatic process.

Unbiased genetic studies in *Drosophila* have identified mutants with extremely low habitual sleep amounts.[37–44] Many of these mutants have reduced rebound, although these results can be difficult to interpret because extreme short sleepers have less sleep to lose during SD.[38–43] Moreover, for at least some short sleepers there is evidence that sleep drive remains high: many of these mutants have an increased number of sleep bouts and

upregulated biomarkers of sleep need.[37,39,41,42,45] Thus, the deficit seems to be in the ability to maintain sleep rather than the ability to sense prior wakefulness. Studying sleep rebound in *Drosophila* may be a more direct way to probe the genetics that underlie the build-up of sleep need.

To date, there is little information on mutants from unbiased screens based on SD, and so the mutations with the most extreme sleep rebound phenotypes following SD have likely not yet been found. Moreover, the relationship between sleep at baseline and sleep during recovery has not been well characterized for either wild-type or mutant *Drosophila*. Thus, it is unclear whether baseline sleep and rebound sleep are closely related across different genotypes or if these two phenomena are largely independent.

In this study we develop a thermogenetic tool for SD in *Drosophila* that enables high-throughput screening to identify lines with reduced sleep rebound. This method produces a strong and consistent sleep rebound compared with other thermogenetic methods, and results in less within-genotype variance compared to sleep rebound following mechanical and caffeine-induced SD. In the course of developing this tool, we find that activation of some populations of neurons produces strong sleep loss with no apparent homeostatic compensation the following day. We used thermogenetic stimulation of a population of neurons that does produce a homeostatic response to perform a screen on a collection of mutant insertion lines generated by the Genome Disruption Project[46,47] and identify two lines with low rebound, reflected by a blunted increase in both sleep amount and depth after SD compared to a control line. Neither line shows evidence of a decrease in the duration, consolidation, or depth of sleep at baseline. Furthermore, statistical analysis

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shows that across our screen data set, genotype can explain much of the variance in recovery sleep that is not explained by linear relationships with baseline sleep parameters. Taken together, these findings suggest that regulation of sleep amount under baseline and recovery conditions can be controlled by independent genetic mechanisms.

Results

Development of a Novel Thermogenetic Method to Induce SD in *Drosophila*

We tested thermogenetic methods of SD to identify an approach that could be used as an efficient screening tool (Figure 2-1A). For the thermogenetic methods, we selected candidate Gal4 drivers thought to express in wake-promoting neurons and used these to drive expression of the heat-sensitive cation channel TrpA1. Candidate Gal4 drivers were selected based on data generated in a recent Gal4 screen for circadian output neurons,[50] in which TrpA1 was used to drive depolarization of Gal4-labeled neurons for 5 days in constant darkness. To assess induced wakefulness and subsequent recovery in these same lines, we employed conditions typically used to study sleep and sleep rebound – 12:12 light:dark cycles (LD) with a single day of deprivation. We crossed candidate lines with Gal4 drivers on chromosomes II or III to lines with a UAS-TrpA1 transgene on the same chromosome. Progeny from these crosses were subjected to a baseline day at 21°C, at which there is no TrpA1 activation,[51] followed by a day at the TrpA1 activation temperature of 28°C, and a subsequent recovery day at 21°C. Sleep loss and sleep rebound were assessed by comparing the 24-h TrpA1 activation and recovery periods with the baseline day.

There is a wide range of effectiveness and consistency in thermogenetically induced

wakefulness across Gal4 drivers (Figure 2-1A). Moreover, drivers that produced

equivalent amounts of sleep loss can produce highly divergent amounts of rebound the

following day. In particular, c584-Gal4, 104906-Gal4, MJ63-Gal4, and c453-Gal4 all

produce substantial sleep loss, but whereas c584-Gal4 and 104906-Gal4 produce

significant rebound, MJ63-Gal4 and c453-Gal4 display little to no evidence of a rebound,

suggesting that these drivers produce wakefulness via a mechanism that circumvents or

counteracts sleep homeostasis.

The Wake-Promoting c584-Gal4 Driver is Expressed in Brain Regions Implicated in Drosophila Sleep

We used c584-Gal4 in subsequent experiments to thermogenetically induce SD because it

produces a consistent rebound and has relatively restricted expression in the fly brain

(Figure 2-1B). We were unable to determine a precise genomic insertion site for the

c584-Gal4 P-element due to the repetitive nature of DNA sequences surrounding the

insertion site (data not shown). However, coupling c584-Gal4 with a UAS-nuclear green

fluorescent protein (nGFP) reporter reveals that c584-Gal4 drives expression in the pars

intercerebralis (PI) and in neurons with projections to the fan-shaped body (Figure 2-1B);

in addition, previous work has shown that c584-Gal4 labels neurons expressing short

neuropeptide F (sNPF).[52, 53] All of these regions have been previously implicated in sleep

control, although the reported roles for the PI and sNPF include both sleep promoting and

wake-promoting functions.[43, 50, 54–56] Previous work identified wake-promoting neurons

with projections to the fan-shaped body in the dopaminergic PPM3 and PPL1 clusters,[57,

58] so we performed experiments to determine whether c584-Gal4 co-localizes with

tyrosine hydroxylase (TH), a marker of dopaminergic neurons. Co-staining brains of

c584-Gal4 > UAS-nGFP animals with the TH antibody reveals overlap between c584

neurons and a subset of dopaminergic neurons in the PPM3 cluster, and close proximity

between c584 neurons and dopaminergic neurons of the PPL1 cluster (Figure 2-1B).

104906-Gal4, although more widespread than c584-Gal4 with staining that appears to

includes Kenyon cells, also labels the PPM3 and PPL1 clusters, making those

dopaminergic clusters good candidates for the wake-promoting effects of these drivers

(Supplemental Figure 2-1). To facilitate screening, we generated a c584-Gal4, UAS-

TrpA1 stock with both transgenes on the same chromosome, into which we could cross

transposon insertion mutations generated by the Gene Disruption Project.[46, 47]

Thermogenetic SD Produces a More Consistent Sleep Rebound With Less Within-Genotype Variance Compared to Caffeine or Mechanical SD

Following development of a thermogenetic method of inducing SD, pilot screens were

conducted using caffeine, mechanical SD, and the c584-Gal4 driven thermogenetic

approach to compare suitability for screening. For the caffeine pilot screen, flies were fed

caffeine at a concentration previously shown to produce sleep loss[59] for 24 h from ZT0-

ZT24, then returned to regular food to assess rebound. For the mechanical SD screen,

flies were sleep deprived by shaking on an adapted vortex for 6 h from ZT18-ZT24. Both

SD protocols were applied to homozygous MiMIC stocks ordered from the Bloomington

stock center. The thermogenetic screening protocol is described in the next paragraph

(Figure 2-2A). For the thermogenetic pilot screen, lethal or second chromosome MiMIC

insertions were tested in the heterozygous condition by crossing the MiMIC stock to the

c584-Gal4, UAS-TrpA1 stock. Importantly, although our c584-Gal4, UAS-TrpA1 line

was backcrossed to an isogenic background, transposon insertion lines generated by the Gene Disruption Project are not generated in isogenic backgrounds, so there may be multiple genetic differences between stocks. Caffeine and mechanical SD pilot screens also allowed for any recessive differences between stocks to be revealed, so the genetic diversity of animals tested in these screens should be greater than the genetic diversity of the heterozygous animals tested in the thermogenetic pilot screen. Despite this, genotype is a stronger determinant of recovery sleep in the thermogenetic pilot screen than either the mechanical SD screen or the caffeine screen (Table 2-1). Moreover, the remaining Root Mean Square Error (RMSE) not explained by genotype is smaller in the thermogenetic screen than the pilot screens with caffeine or mechanical SD. This suggests that rebound following thermogenetic SD presents a more consistent behavior, suitable for genetic screening.

Screen For Mutants With Reduced Sleep Rebound

To ensure that the sleep rebound we measured in our screen was the result of accumulated sleep loss and not an acute response to the retraction of the wake-promoting stimulus, we chose a protocol for screening where SD takes place within the first 9 h of the night (ZT12–ZT21), allowing recovery from the temperature shift to begin 3 h before lights-on (Figure 2-2A). Rebound is defined as the difference in the duration of sleep between the recovery period and the baseline period during the 15 h following SD (ZT21–ZT12), but because most flies sleep through the last 3 h of the night under baseline conditions, a substantial increase in the duration of sleep typically does not occur until the daytime period following SD (ZT0–ZT12). Thus, our protocol favors quantification of residual sleep need that can be attributed to the net sleep loss in sleep

deprived flies. In addition to changes in sleep amount following SD, changes in sleep bout architecture can also be observed; however, these changes are less consistent, with significant heterogeneity across flies (Supplemental Figure 2-2).

The overall screen schematic is presented in Figure 2-2B. We obtained previously mapped in-gene transposon element insertion lines from the MI and KG collections generated by the Genome Disruption Project.[46,47] Both types of transposons are predicted to act as loss-of-function mutations by knocking down gene expression at the site of their insertion. In the primary screen, we tested 1,741 transposon insertion lines, homozygous when possible and heterozygous when the insertion was lethal or on the second chromosome. We focused on lines with reduced rebound as these results were easier to interpret; although we do observe outliers with increased rebound in the screen, these lines tend to have low baseline daytime sleep, creating a greater opportunity to rebound compared to a fly with a more prominent siesta. To identify lines with reduced rebound, we excluded the lines that had high baseline daytime sleep, which created a ceiling effect resulting in lower rebound, and lines in which thermogenetic stimulation did not produce significant sleep loss. Of 1,539 lines that remained, we rescreened lines that fell into the lowest 2.5 percentile in terms of their rebound sleep, approximately 50 min or less (Figure 2-2C). There were two lines, both tested as heterozygotes, for which we were able to recapitulate low rebound below the 10th percentile (~100 min) in four independent experiments: MI00323/+ and MI00393/+ (Figure 2-2D-E).

Lines with Reduced Sleep Rebound Have Normal Baseline Sleep

MiMIC insertions in the two mutant lines, MI00323 and MI00393, were previously

mapped to *Pka-R1*, the regulatory subunit of protein kinase A (PKA), and *N-Cadherin*,[46]

respectively. It should be noted that the PKA pathway has previously been implicated in

sleep maintenance in *Drosophila*.[60] However, preliminary genetic mapping experiments

suggest that the sleep rebound phenotype does not map to the MIMIC insertions

suggesting a contribution of other unknown genetic variations in each of these lines.

If there exists a single homeostatic mechanism that governs sleep need in both

undisturbed conditions and after a perturbation, animals with a reduced rebound might be

expected to have reduced sleep at baseline as well as after SD. However, this does not

appear to be true for the top hits in our screen. During the primary screen in which we

observed reduction in sleep rebound with both MI00323/+ and MI00393/+, overall

baseline sleep duration appears to be similar to all other heterozygous MiMIC insertion

lines tested (Supplemental Figure 2-3).

In order to confirm this observation, we measured the baseline sleep parameters for

MI00323/+ and MI00393/+ alongside MI00386/+, a control MiMIC insertion that

exhibited average amount of rebound in the primary screen. Although baseline sleep is

inconsistent across experiments, we do not observe an overall decrease in baseline sleep

for MI00323/+ and MI00393/+ compared to MI00386/+ (Figure 2-3A). For MI00323/+,

in most experiments there is an increase in daytime sleep amount relative to MI00386/+

that is accompanied by an increase in daytime sleep consolidation, with fewer bouts of

greater length (Figure 2-3B). In MI00393/+, there is a shift in the timing of sleep

compared to MI00386/+, with shorter daytime sleep and longer nighttime sleep accompanied by greater nighttime sleep consolidation. Overall, these findings do not suggest an overall reduction in sleep amount or consolidation at baseline for lines with reduced rebound sleep; rather, baseline sleep appears to be unchanged or increased for these lines relative to the control.

Because daytime sleep amount was sometimes higher in MI00323/+, we wondered if the reduced rebound in this line could be explained by a ceiling effect, wherein this line is unable to recover sleep because baseline daytime sleep is already very high. However, even when compared to the distribution of recovery sleep for all screened heterozygous MiMIC insertion lines with daytime sleep above 300 min, MI00323/+ would still be classified as an outlier (Supplemental Figure 2-4).

Another possible explanation for reduced rebound in our hits is that instead of recovering lost sleep by sleeping longer, these lines recover lost sleep with deeper sleep immediately after SD. To test this hypothesis, we performed an arousal threshold assay at ZT23 for undeprived flies and for flies 2 h following thermogenetic SD. For undeprived flies, there are no significant differences in arousability between MI00323/+, MI00393/+, and the control MI00386/+ (Figure 2-4A). After SD, arousal threshold is increased in all three lines, but this increase is blunted for MI00323/+ and MI00393/+, such that more animals respond to the arousing stimulus. This suggests that sleep depth, like sleep amount, increases less in our hits compared to our control line following SD.

Lines With Reduced Rebound with Thermogenetic SD Do Not Exhibit Reduced Rebound With Mechanical SD

To determine whether the lines identified as hits in our screen exhibit reduced rebound with mechanical SD as well as thermogenetic SD, we subjected heterozygous flies to mechanical SD for 6 h from ZT18–ZT24, as done previously in our pilot screen. In contrast with our findings with thermogenetic SD, we find that sleep rebound is not reduced in these lines following mechanical SD (Figure 2-4B).

Baseline Sleep and Recovery Sleep are Genetically Separable

The data we obtained from the screen for baseline and recovery sleep in ~1,750 lines allowed us to probe the relationships between genotype, recovery sleep, and baseline sleep not just for our hits with the most extreme phenotypes, but also more broadly across the entire screening dataset. We first used a nested ANOVA model with experimental run as a blocking variable to assess the magnitude of the effect of genotype on recovery sleep in our screen. To avoid confounding our subsequent analyses, we used total sleep through the recovery period (not "rebound" as defined to select hits) as the dependent variable in these models. We find in the nested ANOVA that genotype has a significant effect on recovery sleep, with an increase in R^2 of 0.35 when genotype is added ("Reduced Model" in Table 2-2, Figure 2-5).

We next explored the relationship between baseline sleep and recovery sleep in our screen. Most conceptual frameworks for sleep homeostasis predict that baseline sleep and the amount of sleep loss should affect the amount of sleep during recovery, and indeed, there are significant relationships between baseline sleep, sleep loss, and recovery sleep in our data. The relationships between baseline sleep variables and sleep through the

recovery period are adequately described by linear relationships, with both daytime and

nighttime baseline sleep positively correlated with recovery sleep (Table 2-3,

Supplemental Figure 2-5). Sleep through the thermogenetic SD period is, as expected,

negatively correlated with recovery sleep. A square root transformation of the sleep

during thermogenetic deprivation variable produces a better fit than the untransformed

variable, so this transformation is used in this and subsequent models.

A model including all three of these variables (daytime baseline sleep, nighttime baseline

sleep, and sleep through SD) has an R^2 value of 0.255, indicating modest predictive

value. Adding variables reflecting sleep episode length and number at baseline do not

significantly improve the fit of the model, suggesting that these are not meaningful

determinants of recovery sleep in *Drosophila* when sleep amount has already been taken

into account.

Given these relationships between baseline sleep and recovery sleep, two different ways

that genotype might contribute to sleep rebound can be distinguished: (1) by altering the

amount of baseline sleep or sleep loss, secondarily affecting rebound sleep, or (2) by

specifically affecting recovery sleep in a way that is independent from effects on baseline

sleep or sleep loss. As noted previously, baseline sleep for hits MI00323/+ and

MI00393/+ is only minimally different from the control line MI00386/+, and these lines

have near- complete sleep loss, supporting the second possibility. To address whether this

finding in our hits would extend to the entire set of screening data, we constructed a

hierarchical multiple linear regression model that includes predictor variables reflecting

baseline sleep and the amount of sleep through the thermogenetic stimulation (as well as

the potential confounding factor of experimental run, included in the reduced

model/nested ANOVA) and asked whether the effect of genotype persists even when

these variables have been accounted for.

Predictor variables were added to the multiple linear regression model sequentially in the

order listed (Table 2-2). Without genotype, the model with the predictor variables

(baseline sleep, sleep during deprivation and experimental run) has a total R^2 value of

0.308, suggesting these variables could account for a substantial amount of variance in

the data. Most of this effect is due to the correlations with baseline sleep parameters

discussed above, although experimental run also has a significant effect.

When genotype is added to the model that includes baseline sleep and sleep through the

thermogenetic stimulus, the R^2 value increases by 0.24. Compared to 0.35, the change in

R^2 when genotype is added to the reduced model, this is a somewhat smaller effect (Table

2-2, Figure 2-5). This suggests that some of the effect of genotype on recovery sleep can

be thought of as secondary to effects of genotype on baseline sleep or sleep through the

thermogenetic stimulus. Nonetheless, the larger part of the effect of genotype on recovery

sleep persists in the full model. Thus, to the extent that the linear modeling reflects the

true relationship between baseline sleep and recovery sleep in *Drosophila*, our data

support the idea that much of the effect of genotype on recovery sleep is direct and

cannot be explained by indirect effects from relationships with baseline sleep or sleep

loss.

To ensure that the effect of genotype is not due to broad differences in genetic

background resulting from the different collections we screened, but rather to specific

differences between individual lines within a collection of insertions, we applied the same modeling approach separately to each type of insertion we screened (Supplementary Tables 2-1, 2-2, and 2-3). Although the relative contributions of baseline sleep and sleep loss vary among the different collections, in all collections the effect of genotype on recovery sleep persists even when variables reflecting baseline sleep and sleep through thermogenetic SD are included in the model.

Discussion

Sleep homeostasis is often described as a single process that regulates sleep both when animals are left undisturbed and when animals are kept awake for extended periods.[61] Disparate molecules have been implicated in regulating sleep amount and intensity, but this work has not yet yielded a coherent mechanism to explain all aspects of the proposed homeostatic "Process S".[24] A growing body of evidence suggests that responses to SD, sleep restriction, or disruption expose mechanisms regulating sleep homeostasis that are not observed under undisturbed conditions, and conversely there are manipulations that substantially affect daily sleep amount without producing a subsequent homeostatic response. This may explain why attempts to identify a unified molecular mechanism for sleep homeostasis have thus far not been fruitful.

Here, we have developed a thermogenetic method of inducing SD that produces a more uniform response and is more subject to influences from genotype than mechanical or caffeine-based approaches. In the course of developing a thermogenetic method to induce SD, we find that manipulations of some neuronal populations produce strong reductions in sleep followed by a strong rebound, whereas other populations of neurons produce

strong sleep loss without any rebound the next day (Figure 2-1A). This finding is reminiscent of the observation that certain environmental factors are able to provoke changes in habitual sleep amount in organisms without apparent homeostatic compensation. Although these findings have been somewhat controversial, food availability, mating status, light, and seasonal migration have all been reported to suppress sleep without a subsequent rebound.[62–66] Our work suggests that there are neural substrates for wake-promoting mechanisms that are able to bypass or counteract the accumulation of sleep need, which may explain how environmental factors are able to provoke changes in sleep that appear to circumvent a homeostatic response.

We also describe genetic manipulations that specifically affect sleep during recovery from SD but have little apparent effect on sleep at baseline. Our unbiased screen yielded two lines that show no evidence of reduced total baseline sleep, despite having little to no sleep rebound and a blunted increase in sleep depth after SD (Figures 2-2, 2-3, and 2-4). Multiple linear regression analysis of our data suggests that these observations can be generalized to our entire screening data set: although we do observe positive correlations between baseline and recovery sleep, genotype has a strong effect on sleep after SD that is not explained by baseline sleep parameters (Tables 2-2 and 2-3, Figure 2-5).

Despite a robust phenotype with thermogenetic SD, our hits do not show reduced rebound with mechanical SD (Figure 2-4B). Given our results showing that the response to mechanical SD is less susceptible to genetic perturbation (Table 2-1), this is not necessarily surprising. It is possible that mechanical SD invokes multiple neural circuits to produce sleep rebound, whereas our thermogenetic approach invokes a specific

neuronal mechanism. Similar findings have been observed in other organisms; recent work in *Caenorhabditis elegans* shows that distinct genetic mechanisms regulate sleep after strong disruptions compared to microhomeostatic regulation of quiescent bouts under undisturbed or "low-noise" conditions.[67] Taken together, these studies implicate sleep rebound as a phenomenon that is mechanistically distinct from sleep at baseline, and suggest that there are multiple mechanisms that calibrate sleep to different types of environmental conditions and perturbations.

The findings presented here highlight the potential of the model organism *Drosophila* to elucidate mechanisms that underlie sleep and other behaviors. The ability to identify mutants with highly extreme phenotypes in large genetic screens allowed us to identify two lines with little to no sleep rebound following thermogenetic SD that nonetheless exhibit normal sleep at baseline. It is currently unclear whether the phenotypes will map to single genes. Further work will be important to determine whether these animals are sensitive to other behavioral consequences of SD – for example, whether learning and memory is affected in the absence of sleep rebound – or if they are truly resilient. Nonetheless, the lines identified in our unbiased genetic screen demonstrate that extreme phenotypes specific to SD can result from genetic differences between animals, and provide the field with valuable tools for identifying mechanisms that underlie the response to SD.

Figure 2-1: Development of a novel thermogenetic tool to induce sleep deprivation (SD) and rebound in *Drosophila*
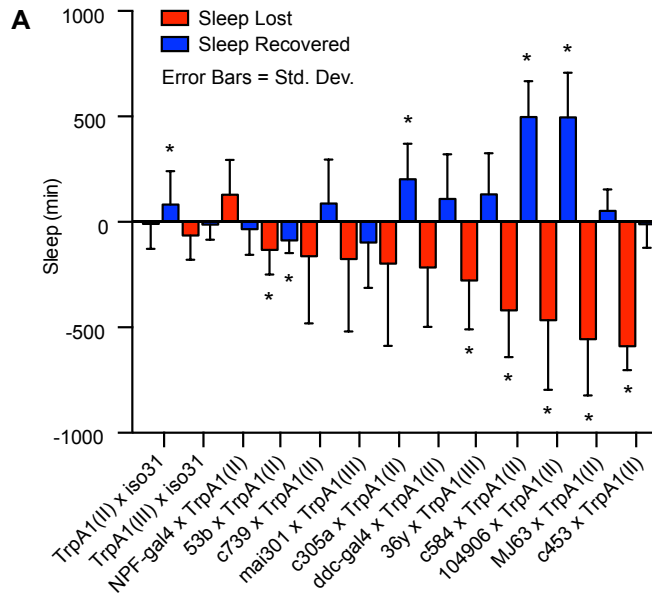
c584-Gal4>UAS-nGFP
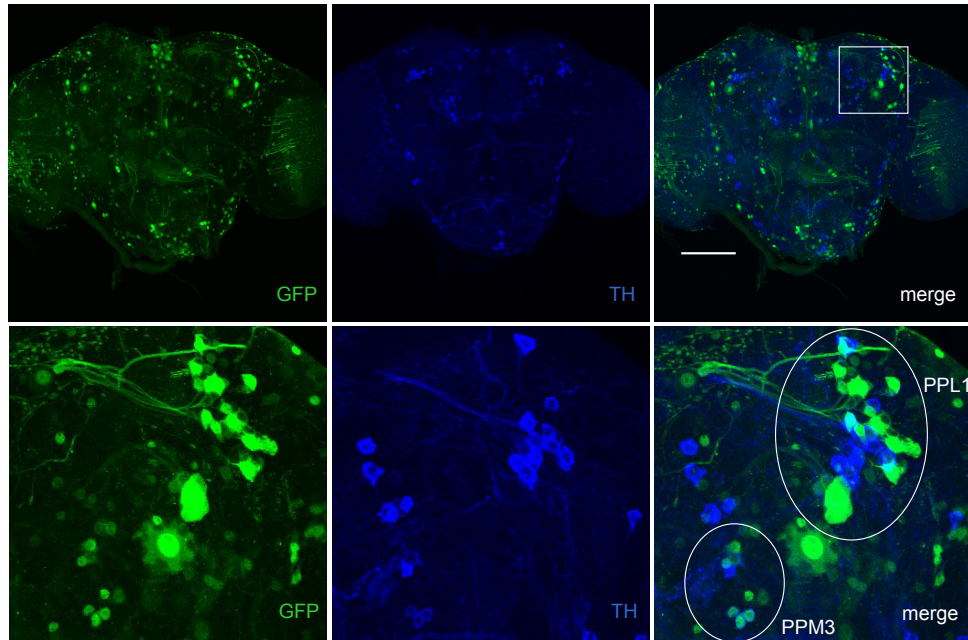
Figure 2-1. Development of a novel thermogenetic tool to induce sleep deprivation (SD) and rebound in *Drosophila* (A) Gal4 lines were screened to identify drivers that produce strong sleep loss and subsequent rebound when coupled with the heat-activated cation channel TrpA1. Each candidate Gal4 driver was paired with a UAS-TrpA1 transgene on the same chromosome as the Gal4 driver. A full day of baseline data were collected at 21°C, followed by 24 h of TrpA1 activation at 28°C (ZT0-ZT24) and a subsequent recovery day where flies were returned to 21°C. Error bars represent standard deviation. Significance was assessed with a one-sample Student *t*-test with a Bonferroni correction for multiple testing. n = 11-52 per genotype. **(B)** GFP expression in c584-Gal4/UAS-nGFP flies shows relatively sparse expression in the brain driven by c584-Gal4. Immunohistochemistry with anti-TH and anti-GFP antibodies reveals clustering and costaining of c584-expressing neurons with dopaminergic neurons. GFP expression in c584-Gal4/UAS-nGFP flies includes non-dopaminergic neurons around the dopaminergic PPL1 cluster and co-staining with TH in 2-3 neurons of the PPM3 cluster. Scale bar = 100 μm.

Figure 2-2: Thermogenetic screen for mutants with reduced sleep rebound

A

Screen Protocol
(21°C)　　　Baseline (21°C)　　　　　Sleep Dep. (29°C)　　Rebound (21°C)　　　　(21°C)

Representative Screening Group:

B

Primary Screen
1741 Transposon Insertion Lines

↓

Excluded
Lines with Low Sleep Loss
High Baseline Sleep (Day)
1539 Lines
Median Rebound: 216 minutes

↓

Candidates for Rescreening
< 2.5 percentile: 50 minutes
38 Lines

↓

Hits
< 10th percentile: 103 minutes
4 Independent Experiments
2 Lines

C

D

E

MI00323/+

MI00393/+
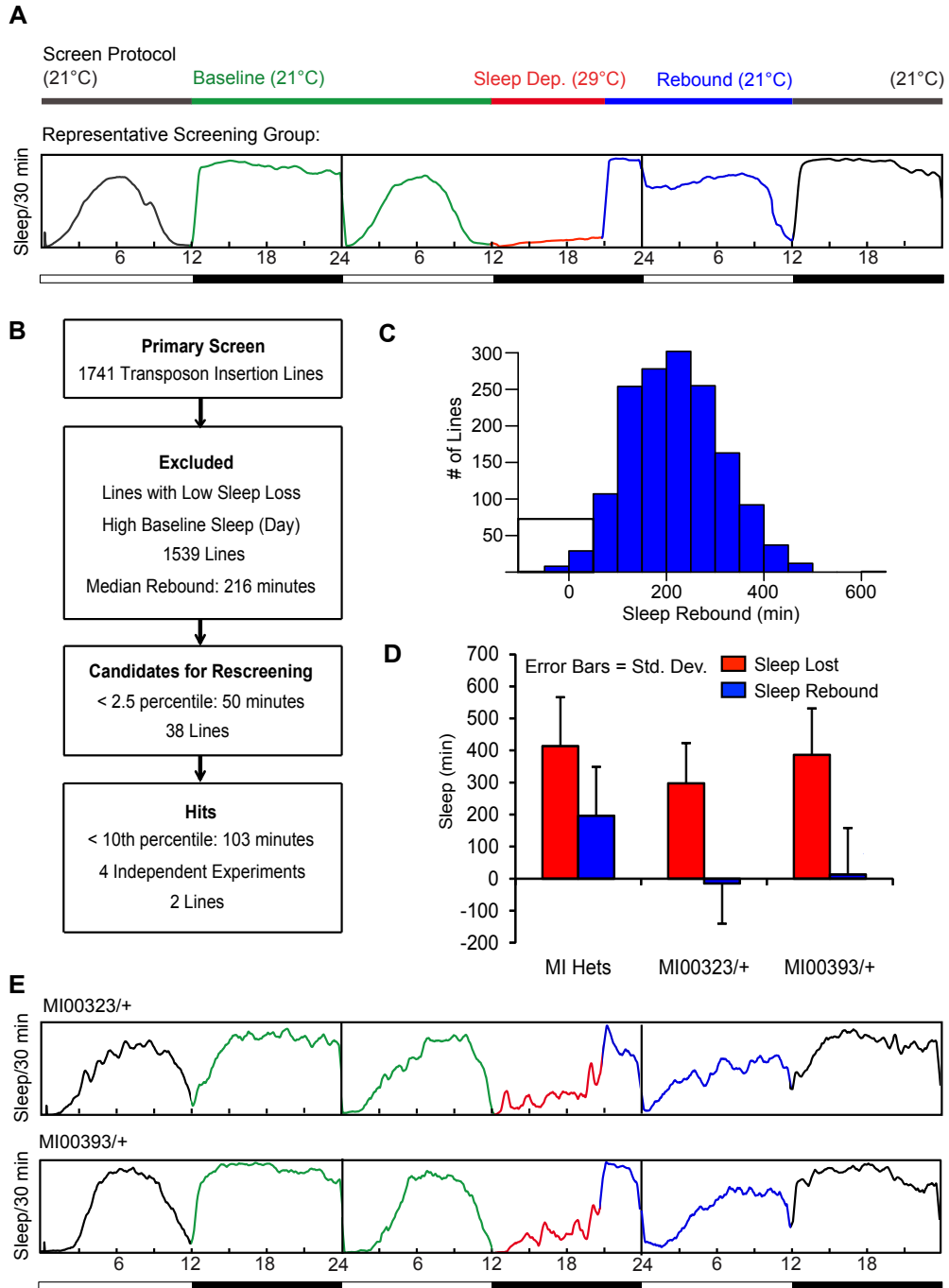
Figure 2-2. Thermogenetic screen for mutants with reduced sleep rebound. (A)

Screen protocol: Insertion lines were crossed into the c584-Gal4, UAS-TrpA1 stock and

the flies were entrained at 21°C. Sleep deprivation (SD) was induced for 9 h (ZT12-

ZT21) at 29°C, after which the flies were allowed to recover at 21°C. The PySolo sleep

profile presented is average sleep of all flies from a representative group of lines run in

the screen. Y-axis represents fraction of time asleep in a 30-min bin. **(B)** Overall screen

schematic: Flow chart describing the number of insertion lines selected at each screening

stage. **(C)** Histogram showing rebound sleep (sleep amount on the recovery day

subtracted from sleep amount on the baseline day Zeitgeber time (ZT)21-ZT12) for all

the lines tested in the screen. Candidates for rescreening (<2.5 percentile) are boxed. **(D)**

Sleep rebound hits: After rescreening, two lines, MI00323/+ and MI00393/+, show

reduced rebound after SD. Plotted are sleep loss (SL) and sleep recovered (SR) from four

independent experiments for MI00323/+ and MI00393/+ compared to all MiMIC

insertions tested as heterozygotes in the screen. Error bars represent standard deviation.

(E) Sleep profile for MI00323/+ and MI00393/+ during the thermogenetic SD protocol in

a representative experiment.

Figure 2-3: Baseline sleep is not reduced in lines with reduced rebound
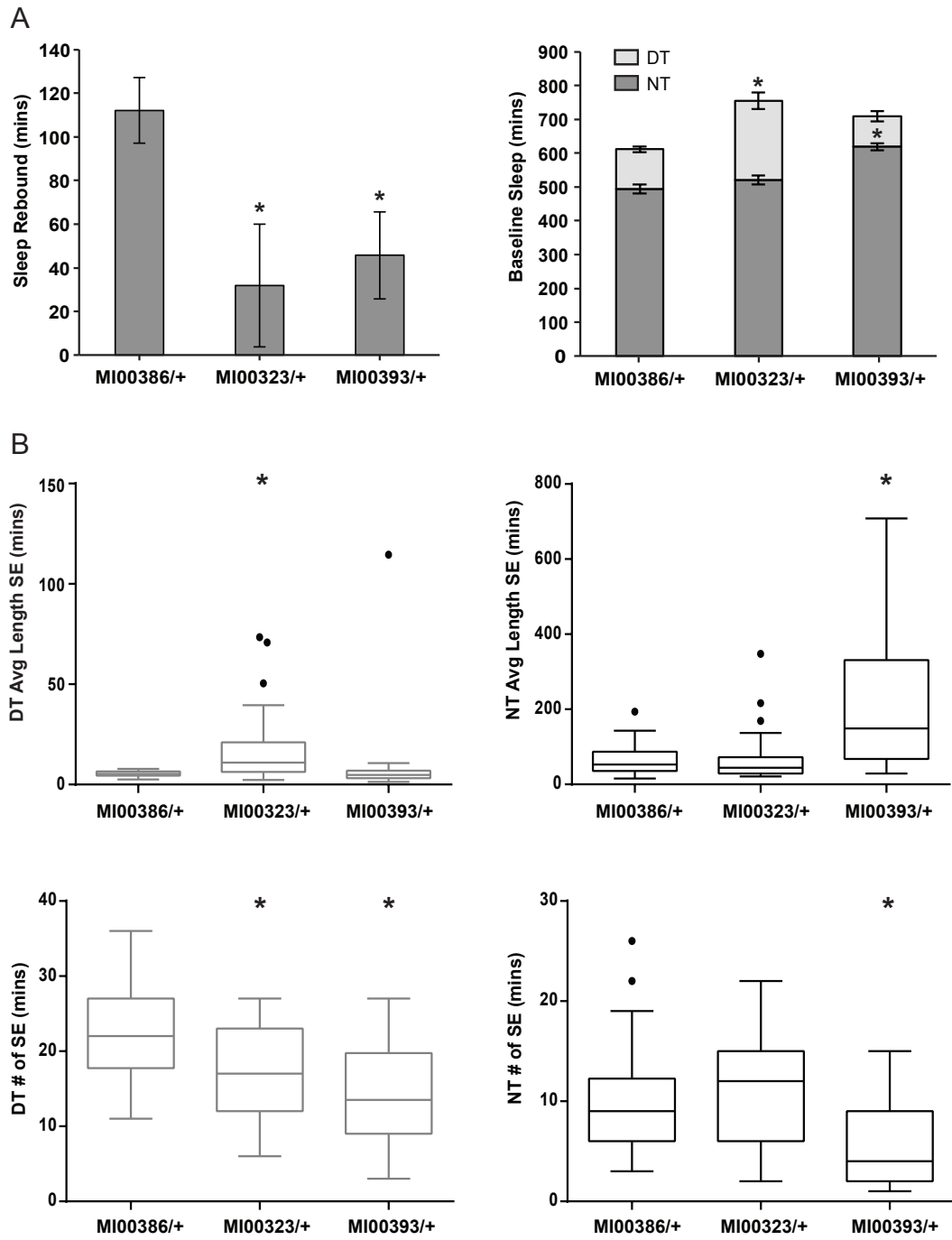
Figure 2-3: Baseline sleep is not reduced in lines with reduced rebound. (A) Sleep

rebound following thermogenetic SD and baseline sleep amount (±standard error of the

mean) and **(B)** sleep episode data for a representative experiment are shown for

MI00323/+ and MI00393/+ (lines with reduced rebound) compared to MI00386/+, which

had an average amount of rebound sleep following sleep deprivation (SD) in the primary

screen. For MI00323/+, rebound was significantly reduced in four of four experiments

with n = 28-32 per genotype. Increase in baseline daytime (DT) sleep episode (SE) length

was significant in five of seven experiments, and increase in baseline DT sleep amount,

and decrease in baseline DT SE number were significant in four of seven experiments.

For MI00393/+, rebound was significantly reduced in three of three experiments.

Increase in baseline nighttime (NT) SE length and decrease in baseline NT SE number

was significant in four of six experiments, and increase in baseline NT sleep amount and

decrease in baseline DT sleep amount (not significant in the representative experiment

shown) were significant in three of six experiments. Significance for sleep rebound and

sleep amount data was assessed with Welch t-test, $P < 0.05$. Significance for sleep

episode data was assessed with Wilcoxon rank-sum test, $P < 0.05$. n = 28-32 per

genotype in each experiment.

Figure 2-4: Reduction in sleep rebound following thermogenetic sleep deprivation (SD) extends to sleep depth/arousal threshold but is not observed following mechanical SD
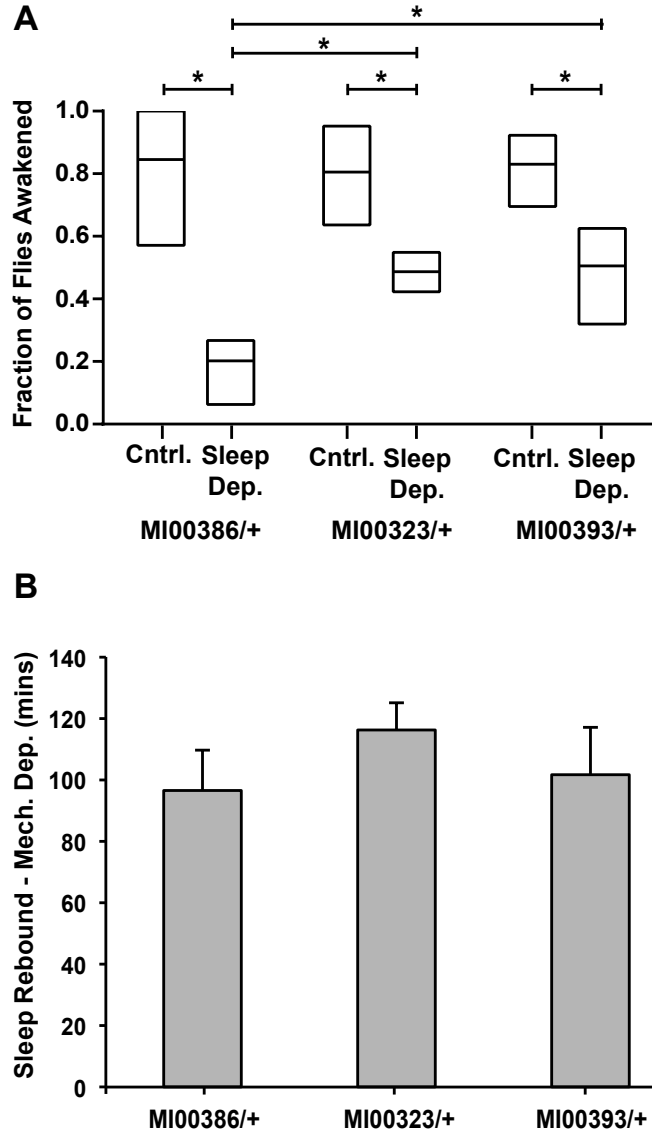
Figure 2-4. Reduction in sleep rebound following thermogenetic sleep deprivation (SD) extends to sleep depth/arousal threshold but is not observed following mechanical SD (A) Arousal threshold for the control MI00386/+ and lines with low sleep rebound, MI00323/+ and MI00393/+, under undeprived (UD) conditions or after thermogenetic SD. Mechanical stimulus was applied at Zeitgeber time (ZT)23, 2 h after the temperature was returned to 21°C for the sleep deprived groups. Flies that were asleep at the time the stimulus was applied were marked as responding if they showed movement within 2 min following stimulus. Plotted data are the mean and range of fraction of flies awoken in four independent experiments (n = 12-32 sleeping flies in each experiment). A two-way analysis of variance with experimental run as an additional blocking variable indicates main effects of SD and genotype on arousal threshold as well as a significant interaction between SD and genotype, $P < 0.05$. Tukey honest significant difference test is used for individual comparisons between groups in *post hoc* analysis. **(B)** Sleep rebound following mechanical SD for the lines identified as hits from the thermogenetic SD screen, with comparison to MI00386/+ as a control. Data are plotted ± standard error of the mean from three combined experiments, n = 24-32 per genotype in each experiment. No significant reduction of sleep rebound is observed for either MI00323/+ or MI00393/+ in three of three experiments assessed by Welch *t*-test, $P < 0.05$.

Figure 2-5: Δ R² in hierarchical multiple linear regression models shows contribution of genotype cannot be explained by effect of genotype on baseline sleep
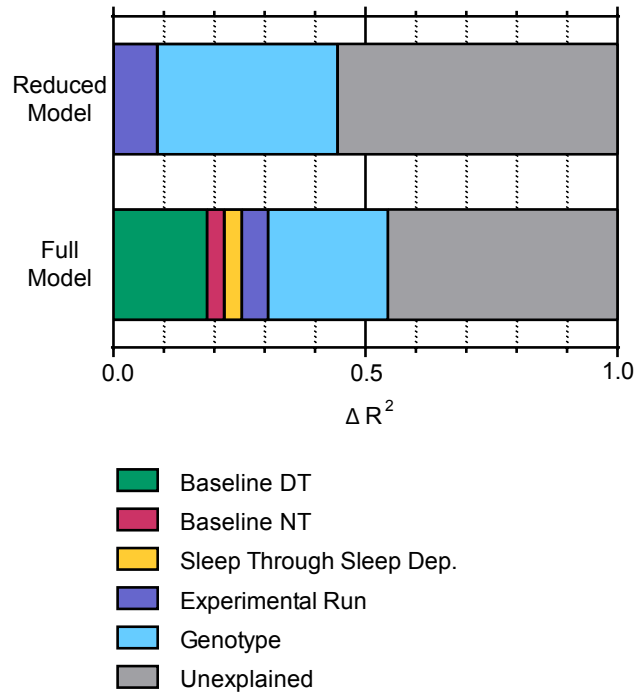
The ΔR^2 is plotted for each variable from both the reduced model and the full model, described in Table 2-2. Variables were added hierarchically to the models in the order depicted (left to right). DT = daytime; NT = nighttime; SD = sleep deprivation.

Table 2-1: Comparison of mechanical, caffeine-induced, and thermogenetic sleep deprivation in pilot screens

	Sleep loss (min)	Sleep reb-ound (min)	RMSE (min)	R^2	Adj. R^2
Mech.	328.3 ± 44.96	219.5 ± 137.29	130.2	0.216	0.100
Caff.	199.3 ± 262.2	255.5 ± 180.75	159.0	0.331	0.226
Therm. SD	387.4 ± 96.47	173.6 ± 153.05	125.3	0.413	0.330

Pilot screens were conducted with mechanical, caffeine-induced and thermogenetic sleep deprivation (SD). Sleep loss and sleep rebound (mean ± standard deviation) for all flies in each pilot screen are reported. The contribution of genotype to sleep rebound was assessed for each screen with a one-way analysis of variance, and root mean square error (RMSE), R^2, and adjusted R^2 values for each pilot screen are reported. In all cases, the effect of genotype was significant at $P < 0.05$. R^2 is greatest and residual RMSE is least with thermogenetic SD, indicating that the thermogenetic approach produces less within-genotype variance compared to the other two approaches and is well suited for genetic screening. Caff. = caffeine induced; mech. = mechanical; therm. = thermogenetic.

Table 2-2: Variance in recovery sleep explained by predictor variables in a hierarchical multiple linear regression model

Model	Variable	RMSE (min)	R^2	Adj. R^2
Reduced model	*Intercept*	163.0	-	-
	Experimental run	156.0	0.087	0.084
	Genotype	130.4	0.444	0.360
Full model	*Intercept*	163.0	-	-
	Baseline DT sleep	147.0	0.186	0.186
	Baseline NT sleep	144.0	0.220	0.220
	Sleep through SD [a]	140.7	0.255	0.255
	Experimental run	135.8	0.308	0.305
	Genotype	117.9	0.546	0.477

[a] Square root transformation.

Two models were used to describe the variance in recovery sleep that could be explained by genotype. The reduced model describes the variance in recovery sleep explained by genotype, correcting for the potential confounding factor of experimental run, but regardless of whether this relationship could be explained if relationships between genotype and other sleep parameters are taken into account. The full model describes the variance in recovery sleep attributed to genotype that cannot be explained by baseline sleep parameters, baseline daytime (DT) sleep or baseline nighttime (NT) sleep, or sleep through the thermogenetic stimulus (sleep through SD). There was evidence for a

nonlinear relationship between sleep through SD and recovery sleep, so a square root

transformation of sleep through SD was used. For both the reduced model and the full

model, variables were added hierarchically in the order listed based on expected

biological relationships. The total root mean square error (RMSE), R2, and adjusted R2

for the models after the addition of each variable are reported. Genotype has a substantial

effect on recovery sleep in both the reduced and full models. All reported variables

significantly improved the model with P < 0.001 (variance ratio test).

Table 2-3: Regression of recovery sleep against daytime and nighttime baseline sleep and sleep through thermogenetic sleep deprivation

Variable	β Coeff. estimate	β Coeff. std. error	P
Intercept	569.3 min	1.22	<0.001
Baseline DT sleep (min)	0.52	0.001	<0.001
Baseline NT sleep (min)	0.43	0.014	<0.001
Sleep through SD [a] $(min^{0.5})$	-5.06 $min^{0.5}$	0.202	<0.001

[a] Square root transformation.

Baseline daytime (DT) sleep, baseline nighttime (NT) sleep, and sleep through the thermogenetic stimulation (sleep through sleep deprivation, SD) were regressed on recovery sleep in a multiple linear regression model. β coefficients and standard error are reported in the table. Positive linear relationships were observed between the amount of baseline sleep and the amount of recovery sleep. A negative relationship between the amount of sleep through SD and the amount of recovery sleep was observed. Relationships are plotted in Supplemental Figure 2-3. There was evidence for a non-linear relationship between sleep through SD and recovery sleep, so a square root transformation of sleep through SD was used.

Supplemental Figure 2-1: Coexpression of 104906-Gal4 with dopaminergic cells
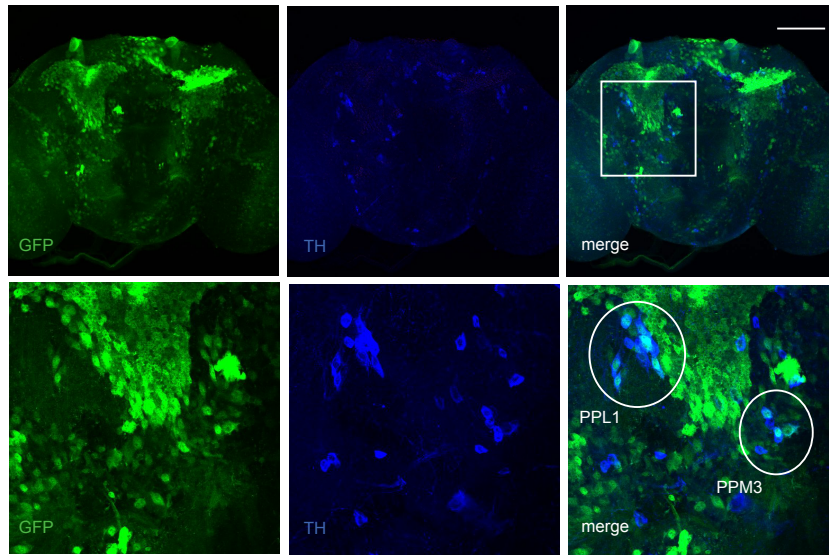
104906-Gal4>UAS-nGFP brains exhibit widespread GFP expression. Costaining with an anti-TH antibody reveals coexpression of GFP and TH in the PPL1 and PPM3 clusters. Scale bar = 100 μm.

Supplemental Figure 2-2: Changes in sleep parameters after thermogenetic sleep deprivation (SD)
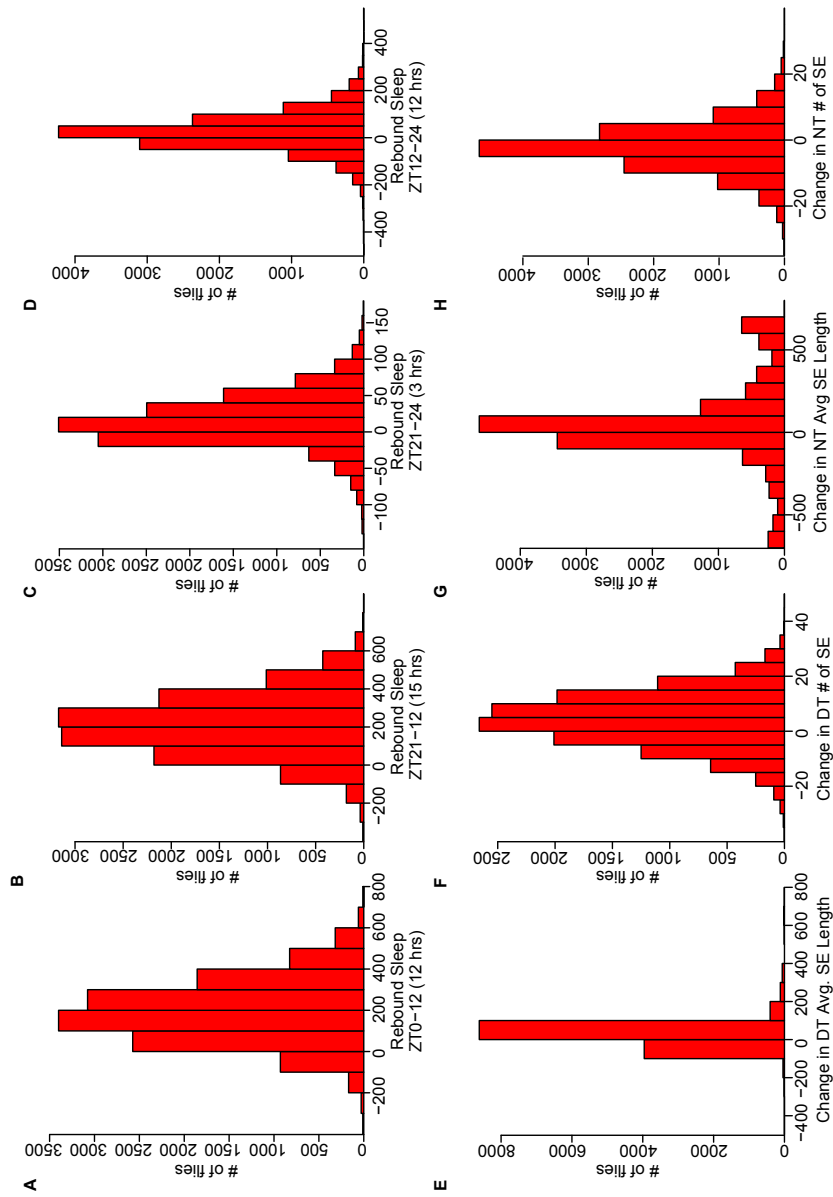
Supplemental Figure 2-2. Changes in sleep parameters after thermogenetic sleep deprivation (SD). (A-D) Histograms showing change in sleep amount for individual flies in the primary screen. An increase in sleep amount (rebound) relative to baseline is observed from Zeitgeber time (ZT)0-12 **(A)** and over the 15-h period from ZT21-12 in almost all flies **(B)**. Net rebound sleep during the 3 h immediately following SD (ZT21-24) **(C)** and the first full night following SD (ZT12-24) **(D)** can also be observed, but are less consistent, with many flies showing the opposite trend. **(E-H)** Histograms showing change in sleep bout architecture for individual flies in primary screen. During the day, flies tend to have longer **(E)** and a greater number **(F)** of sleep bouts following SD. During the following night, sleep becomes more consolidated, with longer bouts **(G)** and fewer of them **(H)**. As with some of the sleep amount parameters discussed here, these trends are observed for the data set as a whole, but are not consistent across individual flies, with many flies exhibiting the opposite relationship.

Supplemental Figure 2-3: Baseline sleep is not reduced in hits with reduced rebound compared to other MiMIC lines tested in the screen
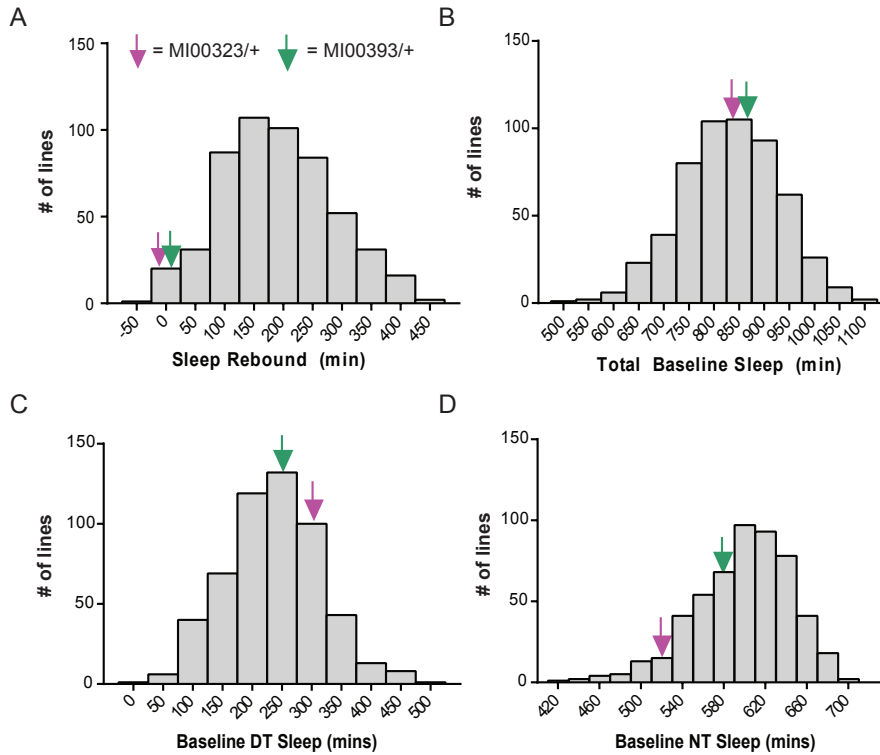
(A-D) Histograms depicting values for (A) sleep recovered (Zeitgeber time 21-12) (B)

total baseline sleep amount, (C) baseline daytime (DT) and (D) baseline nighttime (NT)

sleep for all MiMIC lines run as heterozygotes in the screen. Arrows depict baseline sleep

for MI00323/+ and MI00393/+ in the first four independent experiments for each of these

lines, run in parallel with the primary screen. Overall baseline sleep values for

MI00323/+ and MI00393/+ are within the normal range compared to other lines tested in

the screen. We do observe a shift in the timing of sleep for MI00323/+ toward greater DT

sleep, which we discuss in more detail later. Although in this data set we observe a

reduction in baseline NT sleep for MI00323/+, we do not see significant differences in

baseline NT sleep between this line and a control line discussed in Figure 2-4.

Supplemental Figure 2-4: Histogram of rebound sleep for all heterozygous MiMIC lines that displayed high daytime baseline sleep (>300 min)
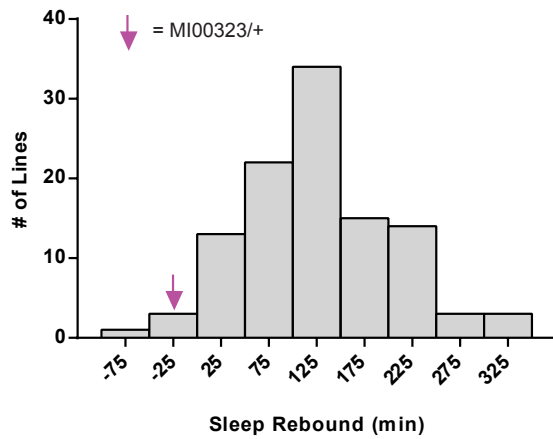
In the primary screen MI00323/+ did not have daytime sleep above 300 min but would have still been an outlier for recovery sleep when compared with other MiMIC insertions with high daytime baseline sleep.

Supplemental Figure 2-5: Relationships of recovery sleep with baseline sleep and sleep through thermogenetic sleep deprivation (SD)
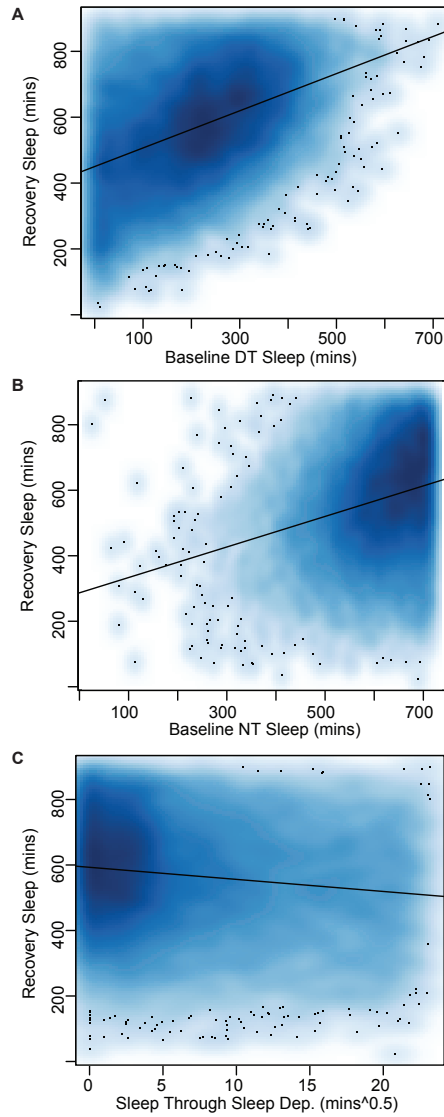
(A-C) Smoothed scatter plots depict relationships between sleep during the recovery period and **(A)** baseline daytime (DT) sleep **(B)** baseline nighttime (NT) sleep and **(C)** square root transformation (see Table 2-3) of sleep through the thermogenetic stimulation (sleep through SD) in primary screen.

Methods

Fly Stocks and Crosses

Fly stocks and crosses were maintained at room temperature or 18°C on standard cornmeal-molasses medium. Mutant lines carrying MI{MiC} ("MI") and P{SUPor-P} ("KG") insertions generated by the Gene Disruption Project were obtained from Bloomington Stock Center at Indiana University. Lines with transposon insertion sites within the body of genes expressed in the central nervous system were selected for screening (Flybase.org). UAS-TrpA1 and MJ63-Gal4 were a gift from L. Griffith. 53b-Gal4 line was a gift from R. Greenspan. c305-Gal4 was a gift from S. Waddell. 36y-Gal4 and NPF-Gal4 were gifts from P. Taghert. c584-Gal4, c739-Gal4, and Ddc-Gal4 were ordered from the Bloomington Stock Center. 103808-Gal4 and 104906-Gal4 lines were ordered from the *Drosophila* Genetic Resource Center. The c584 and UAS-TrpA1 stocks were each outcrossed into an isogenic background, and a c584-Gal4, UAS-TrpA1 stock was made from these outcrossed lines by allowing meiotic recombination in c584-Gal4/UAS-TrpA1 parents. Progeny carrying a recombined chromosome with both transgenes were identified by polymerase chain reaction and then crossed to a balancer stock to generate a stable line.

Sleep Assays

Sleep was monitored using the *Drosophila* Activity Monitoring (DAM) System (TriKinetics, Waltham, MA) in glass locomotor tubes containing 5% sucrose / 2% agarose food. Activity data were collected in 1-min bins. All behavioral experiments were conducted in a 12 h:12 h light-dark (LD) cycle. To test potential thermogenetic

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methods of SD, flies were raised at 18°C until they were 1 to 9 days of age. To test the

effects of thermogenetic neuronal stimulation, flies were loaded into the DAM system

and placed at 21°C, entrained for 2 to 4 days, then subjected to a full day at 28°C starting

at Zeitgeber time (ZT)0. For caffeine-induced SD, flies were raised to 3 to 6 days old at

25°C, then loaded into the DAM system and flipped to food containing 0.5 mg/mL of

caffeine for 24 h starting at ZT0 on day 5. For the pilot mechanical SD screen and

subsequent mechanical SD experiments, flies were raised to 4 to 7 days old at 25°C, then

loaded into the DAM system and sleep deprived from ZT18–24 on day 4 or day 5 by

shaking on an adapted vortex (TriKinetics, Waltham, MA) for 2 sec every 20 sec. In the

primary thermogenetic screen and in subsequent experiments with the c584-Gal4, UAS-

TrpA1 thermogenetic method of SD, transposon insertion lines were crossed into the

c584-Gal4, UAS-TrpA1 background. For heterozygous insertions, progeny of the cross

between the insertion stock and the c584-Gal4, UAS-TrpA1 stock were tested. For

homozygous insertions, balancers were used to track the insertion in two- to three-

generation crossing schemes. For testing responses to thermogenetic SD, flies were raised

at 18°C to 7 to 13 days old, loaded into DAMS tubes, and entrained for 4 days at 21°C.

SD was induced by raising the temperature to 29°C from ZT12–ZT21 on day five. Five

to eight female flies per genotype were tested in the primary screen. Total sleep times

were obtained from DAMS data using PySolo,[48] and sleep consolidation data was

obtained using either PySolo or Excel Macros generated by the Allada laboratory.[49]

Arousal Threshold Assays

For arousal threshold assays, female flies were raised as described previously for the thermogenetic screen and loaded into DAMS monitors. Arousability was assessed at ZT23 for both undisturbed flies, kept at constant 21°C, and SD flies, subjected to 9 h of thermogenetic SD from ZT12-ZT21. The stimulus was generated by dropping a 12 oz. rubber weight from a 4.5-inch height onto the rack supporting DAMS monitors. Sleeping flies, with no activity in the 5 min prior to the stimulus, were counted as aroused if they exhibited beam crossings in the 2 min following the stimulus.

Immunohistochemistry

Fly heads were opened and fixed in 4% paraformaldehyde (in phosphate buffered saline, PBS) for 15–20 min before brains were dissected. All dissection, washing, and immunostaining was done in PBS with 0.1% Triton-X100 (PBS-T). Following dissection, brains were washed three times, incubated 30 min in blocking buffer (5% normal goat serum) and incubated overnight at 4°C in primary antibody solution of 1:300 rabbit anti-tyrosine hydroxylase (TH) AB152 (Millipore, Darmstadt, Germany) and 1:500 chicken anti-green fluorescent protein (GFP) GFP-1020 (Aves Labs, Tigard, OR) in blocking buffer. The following day brains were washed three times, incubated 90 min in secondary antibody solution of 1:400 Alexa Fluor 488 goat anti-chicken and 1:400 Alexa Fluor 680 goat anti-rabbit (Life Technologies, Carlsbad, CA) or 1:400 Cy5 goat anti-rabbit (Rockland Immunochemicals, Pottstown, PA) in blocking buffer, washed three times, then mounted in Vectashield. Brains were imaged on a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany).

Statistics

Statistics were performed using the base package in R version 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria). For multiple linear regression, variables were added to the model hierarchically in a predetermined order based on expected biological relationships. The analysis of variance (*anova*) function was used to perform a variance ratio test comparing each new model to the previous model to assess the significance of the new variables.

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Chapter 3: Reduced levels of the wake-promoting stimulus during sleep loss reduces sleep rebound after thermogenetic sleep deprivation

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Introduction

Sleep rebound, the increased duration and depth of sleep after sleep deprivation (SD), is a common behavior across the animal kingdom, but the molecular mechanisms that produce it are still not understood. There is evidence that sleep rebound is mechanistically distinct from baseline sleep and that different types of SD produce different magnitudes of sleep rebound (Kalinchuk *et al.* 2008; Halassa *et al.* 2009; Seugnet *et al.* 2011; Suzuki *et al.* 2013; Driver *et al.* 2013; Nagy *et al.* 2014; Thimgan *et al.* 2015; Seidner *et al.* 2015; Dubowy *et al.* 2016; Liu *et al.* 2016), and a number of genes and pathways, discussed below, have been implicated in this behavior. However, a unified mechanism for sleep rebound remains elusive.

In mammals, mechanistic studies of sleep rebound have historically focused on the role of potential somnogens – neurochemicals that increase globally during wake and are capable of inducing sleep. While several neurochemicals, including adenosine, tumor necrosis factor-α, and prostaglandin D, meet these criteria, genetic studies related to these chemicals produce only mild phenotypes (Mizoguchi *et al.* 2001; Deboer *et al.* 2002; Stenberg *et al.* 2003; Huang *et al.* 2005; Bjorness *et al.* 2009). In *Drosophila*, sleep rebound is often studied using mechanical SD, which shares features with other sleep-inducing stress response pathways (Toda and Sehgal, unpublished). Several categories of genes have been implicated in sleep rebound – in particular, mutations that affect cellular stress or lipid metabolism modulate sleep rebound after mechanical SD (Shaw *et al.* 2002; Naidoo *et al.* 2007; Thimgan *et al.* 2010; 2015). There is also evidence that changes in synaptic strength in the ellipsoid body R2 neurons may be important for the response to mechanical SD, although the relevance of these neurons to other sleep

behaviors and connections to other sleep regulatory centers are unclear (Liu *et al.* 2016).

Finally, in both *Drosophila* and in mammals, glia may play an important regulatory role

in responses to sleep loss (Halassa *et al.* 2009; Seugnet *et al.* 2011; Dissel *et al.* 2015).

Each of these findings provides compelling clues about the nature of sleep rebound

following mechanical SD, but the connections between these findings, and whether the

same molecular mechanisms are invoked when sleep loss is produced by other means,

both remain unanswered questions.

While these studies focused on mechanical SD, in Chapter 2, I developed a lower

variance method of SD that uses the heat-activated cation channel TrpA1 to

thermogenetically activate wake-promoting neurons labeled with the c584-Gal4 driver

(Dubowy *et al.* 2016). This Gal4 driver labels the wake-promoting dopaminergic PPM3

neurons, among other cells, which are thought to inhibit the sleep-promoting ExFl2

dorsal fan-shaped body cells to produce wake (Ueno *et al.* 2012). Thermogenetically

activating c584-labeled cells produces strong sleep loss, followed by a robust rebound the

following day. I conducted an unbiased genetic screen using this method of SD and

identified two mutant *Drosophila melanogaster* lines that exhibit normal baseline sleep

and sleep loss but little to no sleep rebound.

In this chapter, I follow up on one of the identified hits from the screen and identify a

molecular mechanism responsible for the phenotype. Traditional genetic mapping

experiments did not reveal a single locus responsible for the phenotype, suggesting that

the phenotype is multigenic. However, gene expression studies show that this mutant line

has reduced expression of the enzyme *Dopa decarboxylase* (*Ddc*), an enzyme necessary

for dopamine synthesis, and hypomorphic alleles of *Ddc* partially phenocopy the mutant. The mutant line also has reduced Gal4-driven expression of *TrpA1*. Genetic manipulations that similarly reduce Gal4-driven *TrpA1* expression via Gal4 dilution completely abolish sleep rebound with comparatively modest effects on sleep loss. Taken together, these two findings suggest that the strength of the wake-promoting stimulus is a determinant of subsequent sleep rebound even when the amount of sleep loss is largely unaffected.

Results

MI00393 Phenotype Maps to Chromosome II But Not to the Transposon Insertion
The initial genetic screen was conducted using lines from the Gene Disruption Project with known transposon insertions that had been mapped by inverse Polymerase Chain Reaction (PCR) to precise locations in the genome (Bellen *et al.* 2011; Venken *et al.* 2011). However, these lines carry other mutations. This is in part because creation of these lines involved a series of crosses using several different non-isogenized genetic backgrounds (H. Bellen, personal communication), but also because transposon mutagenesis often involves multiple insertion and excision events, which can leave a small insertion or deletion undetectable by inverse PCR (Metaxakis *et al.* 2005). Indeed, lines generated by the Gene Disruption Project are known to carry second site mutations that produce other phenotypes, such as recessive lethality (Venken *et al.* 2011). Thus, mapping is still necessary to determine if the phenotype is caused by the transposon insertion.

Initial mapping experiments with the two hits identified in Chapter 2 were conducted to determine whether the phenotypes map to the chromosome carrying the transposon insertion (Figure 3-1). Fly stocks were created with the chromosome carrying the insertion from the original Gene Disruption Project stock and all other major chromosomes from our wild-type iso31 background. For MI00323/+, the phenotype does not map to the chromosome on which the MiMIC construct is inserted (data not shown). However, the MI00393/+ phenotype does partially map to the chromosome carrying the insertion, chromosome II. Indeed, the line with chromosome II isolated in an otherwise iso31 background demonstrates the same principles observed the original screen – baseline sleep for this line is nearly identical to baseline sleep of the iso31 wild-type controls, but rebound sleep after thermogenetic SD is greatly reduced. The similarities in baseline sleep between chromosome II-isolated MI00393/+ line and the wild-type iso31 line made it easier to link any observed molecular changes to the reduced rebound phenotype, so this line, hereafter referred to as "MI00393/+ (II)," is used in further work, with the wild-type iso31 stock serving as a control.

Although the MI00393/+ phenotype can be mapped to chromosome II, the transposon insertion itself is dispensable for the phenotype (Figure 3-2). To determine the effect of the transposon insertion, a fly line with a precise excision of MI00393 was created and compared to a control that had undergone the same series of crosses but retained the MiMIC insertion. Excision and control lines were generated by crossing the MI00393 Gene Disruption Project line to a line with a heat shock-driven Minos transposase and exposing the progeny of this cross to heat shock during gametogenesis to induce transposase expression (Metaxakis *et al.* 2005). As was done to map the MI00393

phenotype to chromosome II, the excision and control lines went through a series of crosses to create a line where chromosome II originated from transposase-exposed MI00393 flies and the other major chromosomes originated from iso31. Response to thermogenetic sleep deprivation for these lines was then assessed. The control and precise excision lines both have similarly reduced rebound sleep after thermogenetic sleep deprivation, demonstrating that the transposon insertion is not necessary for the phenotype.

Transcriptional Profiling of MI00393/+ and Wild-type Flies Before and After Sleep Deprivation using RNA-Seq

Gene expression profiling using RNA-Seq was undertaken with two goals: first, to compare SD-induced gene expression changes in flies that do and do not experience sleep rebound, and second, to identify molecular lesions – point mutations or gene expression differences – that might be causally responsible for the MI00393/+ mutant phenotype. Gene expression profiling was done on brains of wild-type iso31 and MI00393/+ (II) flies with c584-Gal4 and UAS-TrpA1 with or without exposure to 11.5 hours of heat to produce thermogenetic SD (Figure 3-3). Brains were dissected at ZT0, 30 minutes after thermogenetic SD ceased for the SD group. Importantly, at the time of dissection, there was no difference in sleep/wake history between sleep-deprived flies of the two genotypes, although differences in sleep/wake behavior would have been immediately apparent had the flies been left undisturbed. Therefore, any differences in transcript expression are the result of differences in response to SD or differences in sleep pressure, not differences in behavioral state.

There were many gene expression changes with SD in wild-type flies (Figure 3-3, Table 3-1), most falling into similar categories previously shown to change after SD (Table 3-2): genes involved in synaptic function, second messenger signaling pathways, RNA and protein metabolism, and cellular stress (Cirelli *et al.* 2005; Zimmerman *et al.* 2006; Williams *et al.* 2007; Mackiewicz *et al.* 2009). While the gene expression profiles in some ways resemble those of sleeping flies – in the upregulation of genes involved in macromolecule metabolism – the upregulation of cellular stress response genes resembles gene expression changes in waking or sleep deprived flies. These mixed results are likely the result of the behavioral state at the time these experiments: flies were taken for dissection 30 minutes into the rebound period after 11.5 hours of thermogenetic sleep deprivation. Thus, it is perhaps unsurprising that the expression profile would reflect both sleeping and waking states. Note that while many genes fall into functional categories previously observed to change with sleep or sleep deprivation, we cannot exclude a role for temperature in the observed gene expression changes.

The vast majority (91%) of the 2109 genes that change with SD in wild-type flies also change in MI00393/+ (II) flies, with only 183 genes that change exclusively in wild-type flies (Figure 3-3, Table 3-3). Interestingly, however, there is a large set of genes – 907 total – with expression that is significantly changed with SD in MI00393/+ (II) flies but not in wild-type flies (Figure 3-3, Table 3-4). Most of these genes are upregulated with sleep deprivation and many fall in functional categories related to mitochondrial function, including metabolic activities associated with mitochondria but also including mitochondrial ribosomal proteins and inner and outer membrane mitochondrial transporters (Table 3-5). However, follow up experiments did not indicate a role for

mitochondrial biogenesis in sleep regulation, so the functional significance of these changes remains unclear.

The Illumina sequencing data revealed many genomic polymorphisms that differed between the wild-type iso31 and MI00393/+ (II) lines, with 24,776 total variants mapping to chromosome II. The large number of variants prevented identification of genome-level changes responsible for the phenotype, and recombination mapping likewise failed to identify a single genomic locus that produced the reduced rebound phenotype (data not shown). However, there are relatively few gene expression differences between these genotypes that reached statistical significance in the undeprived groups, even with a liberal False Discovery Rate of 0.40 (Table 3-3, Figure 3-4). Among these baseline gene expression changes, *Dopa decarboxylase* (*Ddc*), an enzyme necessary for dopamine and serotonin synthesis, is notable given that dopamine is likely responsible for the wake-promoting effects of thermogenetic sleep deprivation with c584-Gal4. *TrpA1* expression is lower in MI00393/+ (II) flies; however, with a q-value of 0.42 this missed the criteria for significance, and it was not clear whether this change in expression level reflected endogenous *TrpA1* or Gal4-driven *TrpA1*. To address both these questions, c584-Gal4 was used to drive GFP expression and quantitative PCR (qPCR) was used to assess GFP transcript levels in MI00393/+ (II) flies and controls. GFP cannot be confused for an endogenous transcript and thus provides a direct measure of Gal4-driven expression (Figure 3-4B). This experiment demonstrated that GFP levels are lower in MI00393/+ (II) flies compared to control, indicating that Gal4-driven expression is lower in MI00393/+ (II) flies.

Sleep Rebound after Thermogenetic Sleep Deprivation is Modulated by the Strength of the Thermogenetic Stimulus

Based on the gene expression experiments, I hypothesized that reduced levels of *Ddc* might be responsible for the phenotype of MI00393/+ mutants. To directly test the effects of *Ddc* on sleep rebound after thermogenetic sleep deprivation, null (Ddc^{27}) and hypomorphic (Ddc^{lo}) alleles of *Ddc* were obtained (Wright *et al.* 1982) and both alleles were backcrossed into the wild-type iso31 background. Both Ddc^{27}/+ and Ddc^{lo}/+ have reduced rebound after thermogenetic sleep deprivation (Figure 3-5), although the phenotype is less extreme than the MI00393/+ (II) phenotype. Since *Ddc* is necessary for dopamine synthesis, this finding provides a link between the wake-promoting stimulus used to produce sleep loss and subsequent sleep rebound. However, sleep loss is not reduced in these mutants, suggesting that while the reduction of *Ddc* activity is substantial enough to affect sleep rebound it is below the threshold that would be necessary to reduce sleep loss.

In attempting to confirm the *Ddc* phenotype and localize the effect to c584-labeled neurons, a UAS-*Ddc*-RNAi construct was identified that has a modest effect on sleep loss but completely abolishes sleep rebound after thermogenetic sleep deprivation; unexpectedly, however, a UAS-GFP control made using the same VALIUM vector (Perkins *et al.* 2015) produces a similar phenotype (Figure 3-6). Our earlier observation that Gal4-driven expression is reduced in MI00393/+ (II) flies raised the possibility that the effect of the VALIUM UAS constructs on sleep rebound might be due to Gal4 dilution; that is, that competition between UAS-TrpA1 and the additional UAS construct for a limited amount of Gal4 results in less driven TrpA1 expression, which might

produced the reduced rebound phenotype. Indeed, qPCR confirms that *TrpA1* expression in c584-Gal4/+, UAS-TrpA1/+ flies is much lower in flies with UAS-RNAi or UAS-GFP compared to flies without the additional UAS. Although unexpected, this finding provides additional evidence that the strength of the wake-promoting stimulus is important for subsequent sleep rebound, and like the findings with the *Ddc* alleles, this result suggests that a reduction in the wake-promoting stimulus can produce a substantial effect on sleep rebound that is disproportionate to the effect on sleep loss.

Discussion

Dopa decarboxylase and *TrpA1* link sleep rebound to the neurochemical mechanisms of sleep loss

In this work, I follow up on a previous finding that MI00393/+ flies have reduced sleep rebound after thermogenetic sleep deprivation. I find that the transposon insertion in this line is dispensable for the phenotype, indicating a contribution of second site mutations. Although a specific mutation in MI00393/+ flies that produces the reduced rebound phenotype could not be located, gene expression profiling using RNA-Seq allowed for the identification of two gene expression changes – reduced levels of *Dopa decarboxylase* (*Ddc*) and reduced levels of Gal4-driven *TrpA1* – that appear to be responsible for the reduced sleep rebound of this line. Mutations that reduce levels of *Ddc*, an enzyme necessary for dopamine and serotonin synthesis, specifically reduce sleep rebound following thermogenetic sleep deprivation. Likewise, reduced Gal4-driven expression of *TrpA1* abolishes sleep rebound with comparatively mild effects on sleep loss.

These results support a model where wake-promoting neurotransmitters serve not only to produce sleep loss, but also act as a signal that contributes to the accumulation of sleep need and promotes sleep rebound. One way this might occur is if elevated levels of the wake-promoting neurotransmitter used to produce SD trigger homeostatic plasticity within the sleep-regulating circuit. Indeed, homeostatic plasticity at synapses is a wide spread phenomenon (reviewed in Turrigiano 2012), and thus it would not be unexpected if it occurred in sleep circuits. With the likely dopamine-mediated method of sleep deprivation used here, homeostatic plasticity could take the form of reduced dopamine release, reduced postsynaptic sensitivity to dopamine, or altered intrinsic electrical properties in downstream neurons; in fact, all of these changes have been observed in mammalian circuits in response to perturbations of dopamine signaling (Zigmond 1997; Jones *et al.* 1998; Bezard *et al.* 2003; Perez *et al.* 2008; Azdad *et al.* 2009; Bergstrom *et al.* 2011; Friedman *et al.* 2014; Fieblinger *et al.* 2014).

Although the findings regarding *Ddc* and Gal4-driven *TrpA1* expression together support a model where dopamine is important both for sleep loss and for subsequent sleep rebound, it is important to note that we cannot exclude a role of serotonin, which is also synthesized by *Ddc*, or c584-driven expression of *TrpA1* outside dopaminergic cells in mediating the observed phenotypes. Further work will be conducted to localize the effects specifically to dopaminergic neurons, including work with more restricted Gal4 drivers that express specifically in the relevant dopaminergic cells.

Dopaminergic signaling and mitochondria

Increased expression of mitochondrial genes with sleep deprivation in MI00393/+ (II) flies seems to suggest an increase in mitochondrial biogenesis. There are many connections between dopamine, dopamine signaling, and mitochondrial regulation and function. However, follow-up experiments to directly test the effects of mitochondrial biogenesis on sleep behavior did not reveal any differences in sleep in flies with upregulated mitochondrial biogenesis. Thus, it remains unclear if mitochondrial biogenesis has any functional consequences for sleep rebound, or if it is a secondary result of perturbed dopamine signaling that does not influence sleep behavior.

The relationship between dopamine and mitochondria is multi-faceted. Monoamine oxidase, a gene necessary for the catabolism of dopamine, is located on the mitochondrial outer membrane, and dopamine and its metabolites dose-dependently inhibit electron transfer chain function (Przedborski *et al.* 1993; Ben Shachar *et al.* 1995; Cohen *et al.* 1997; Berman and Hastings 1999; Cohen and Kesler 1999; Khan *et al.* 2005; Gautam and Zeevalk 2011). Mitochondria have also been implicated in the pathogenesis of genetic parkinsonism, a neurodegenerative disorder of dopamine neurons (reviewed in Cookson 2012). The parkinsonism-related genes *PINK1* and *Parkin* act in the same pathway to limit mitochondrial fusion (Clark *et al.* 2006; Park *et al.* 2006; Poole *et al.* 2008; Yang *et al.* 2008; Deng *et al.* 2008), and promote mitophagy (Narendra *et al.* 2008; Vives-Bauza *et al.* 2010; Narendra *et al.* 2010; Vincow *et al.* 2013). It has been suggested that the reason parkinsonism selectively affects dopamine neurons is because the metabolic byproducts present a form of toxicity, in part via oxidative stress, which may increase the

need for efficient mitochondrial turnover (reviewed in Cookson 2012; Goldstein *et al.* 2014).

Dopamine also appears to have non-cell-autonomous effects on mitochondrial function through dopamine receptor signaling. Recent work in *Drosophila* suggests that a dramatic increase in energy metabolism in mushroom body neurons is necessary to support long-term olfactory memory in flies (Plaçais *et al.* 2017). Both this increase in energy metabolism and consolidation of olfactory memory are dependent on signaling from the *Dop1R2* dopamine receptor. A role for *Dop1R2* in mitochondrial regulation is also supported by the finding that *Dop1R2* signaling modulates sensitivity to paraquat, a chemical source of oxidative stress (Cassar *et al.* 2015).

The data discussed above all seem to suggest that flies with greater dopamine synthesis would also have greater need for mitochondrial synthesis or turnover; however, in our data it is the mutant flies with reduced levels of *Ddc* and thus likely reduced dopamine signaling that have apparently increased mitochondrial biogenesis after sleep deprivation. This suggests a complex regulatory relationship. If the hypothesis that in wild-type flies but not MI00393/+ flies there is homeostatic compensation in the circuit in response to persistent dopamine signaling is correct, perhaps that compensation also prevents the need for or execution of a mitochondrial response to dopamine. It is also possible that *Ddc* and mitochondrial biogenesis are regulated by common factors. However, the reduction of *Ddc* in MI00393/+ flies is observed under baseline conditions, while the increase in mitochondrial biogenesis is specifically triggered by thermogenetic sleep deprivation, so this explanation seems less likely.

The role of gene expression changes in rebound after sleep deprivation

Previous gene expression studies have characterized changes that occur with sleep deprivation in wild-type animals (Cirelli *et al.* 2005; Zimmerman *et al.* 2006; Williams *et al.* 2007), but the functional significance of these changes has remained unclear. Our findings show that the vast majority of gene expression changes that occur in wild-type animals also occur in mutants that do not undergo rebound sleep. This does not exclude a functional role for these genes in sleep homeostasis – it is possible that the genes that change with sleep deprivation still represent somnogens and MI00393/+ (II) flies are deficient in pathways that sense and respond to such molecular signatures of sleep loss. Nonetheless, it is interesting that these changes occur even when rebound sleep does not, suggesting that they do not depend on the "rebound" brain state.

Gal4 dilution as a potential confounding factor in *Drosophila* experiments

In this study, we unexpectedly find that introducing a UAS-construct that should have no effect on neuron physiology (UAS-GFP) affects c584-Gal4, UAS-TrpA1-mediated thermogenetic sleep loss and subsequent rebound. Follow-up experiments showed that the observed phenotype is likely due to the fact that upon introduction of a second UAS construct, the available Gal4 transcription factor is now split between two UAS sites, resulting in a dilution of the transcription-promoting effect. This finding underscores the importance of running appropriate controls in Gal4/UAS experiments, especially in experiments with complicated designs that involve multiple transgenes. A few common types of experiments may be particularly vulnerable to erroneous conclusions that result

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from Gal4 dilution; these include 1) experiments where a UAS-transgene is used to rescue a UAS-RNAi phenotype in order to show that the phenotype is not due to an RNAi off-target; 2) experiments where a UAS-RNAi is used to show that a gene is necessary for a change in physiology that is measured with a UAS-driven fluorescent or luminescent indicator; and 3) experiments, like the one described here, that use UAS-RNAi to show that a neurotransmitter or signaling pathway is responsible for a phenotype produced by thermogenetic or optogenetic stimulation of the same neurons. While many experiments in the literature include appropriate controls or screen many UAS lines to identify individual lines with a phenotype, others do not, indicating that the potential for Gal4 dilution to lead researchers to incorrect conclusions is not yet widely appreciated. Controls designed to rule out the possibility of Gal4 dilution should consist of similar UAS constructs, i.e. UAS constructs with the same vector and landing site. Indeed, while the data presented here show Gal4-dilution-related phenotypes with 10X-UAS VALIUM vectors, 5X-UAS vectors did not have the same effect.

Conclusions

In this work, reduced *Ddc* levels were identified as a mechanism for the reduced rebound after thermogenetic sleep deprivation in MI00393/+ mutants. Although baseline sleep and sleep loss are unchanged or increased in MI00393/+ and *Ddc* mutants, sleep rebound after thermogenetic sleep deprivation is reduced. Since wake-promoting dopaminergic cells are thermogentically activated in our method of sleep deprivation, and *Ddc* is necessary for dopamine synthesis, this finding suggests a connection between the extent of dopamine signaling during sleep deprivation and rebound the following day. I also identify reduced Gal4-driven *TrpA1* levels as an additional mechanism that contributes to

the reduced rebound following thermogenetic sleep deprivation in MI00393/+ mutants. A manipulation that reduces TrpA1 levels has modest effects on the extent of sleep loss but abolishes sleep rebound. This work suggests that the strength of the wake-promoting stimulus is a major contributor to sleep rebound following sleep deprivation. The next chapter will discuss experiments to test a model based on these findings, that homeostatic plasticity within sleep regulatory circuits underlies sleep rebound following sleep deprivation.

Figure 3-1: MI00393/+ Phenotype Partially Maps to Chromosome II
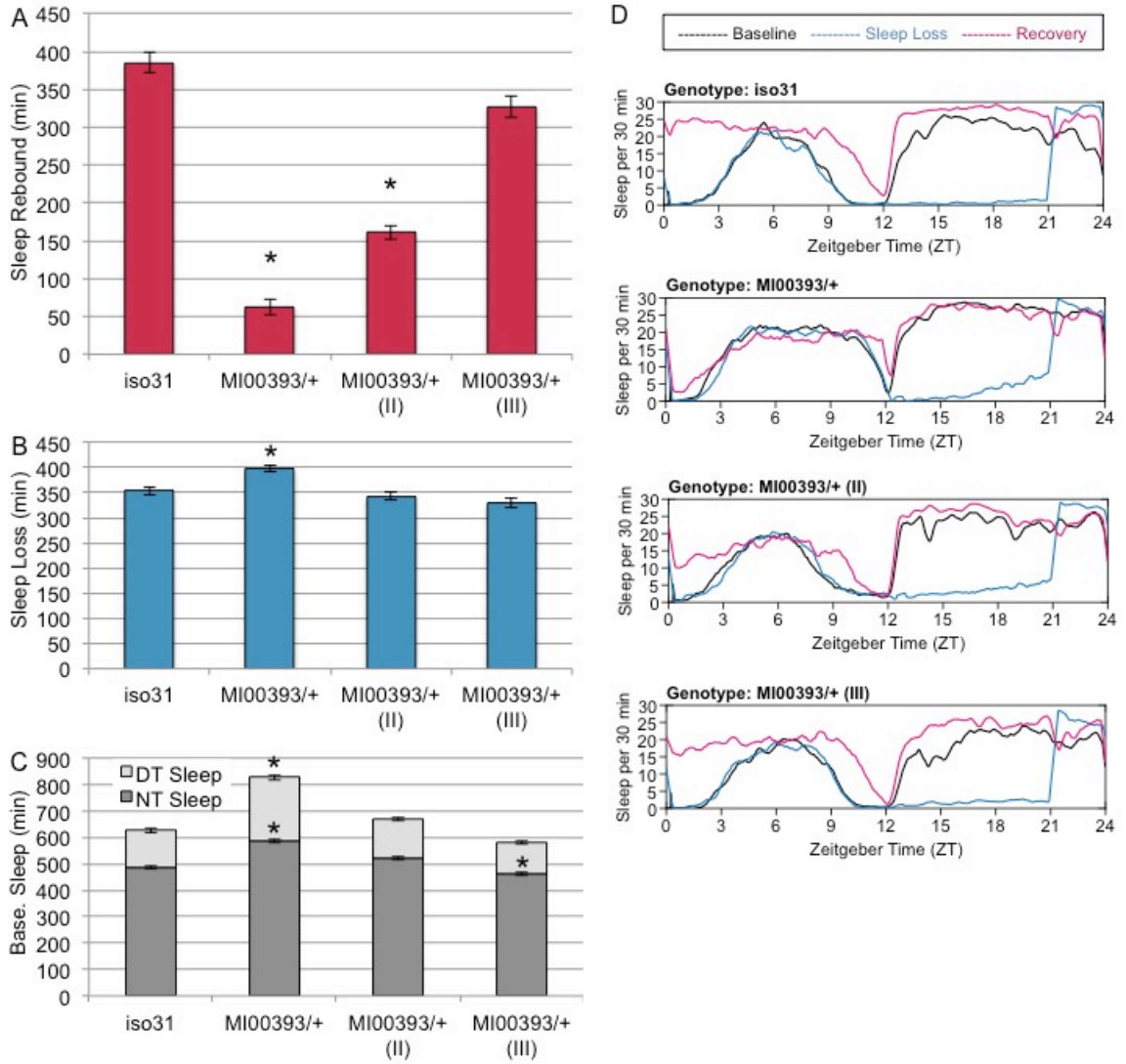
Figure 3-1: MI00393/+ Phenotype Partially Maps to Chromosome II

Thermogenetic sleep deprivation experiment with lines where the MI00393

chromosomes II or III are isolated in an otherwise iso31 background. All genotypes

include c584-Gal4/+, UAS-TrpA1/+. Baseline data is collected at 21°C and sleep

deprivation is produced by exposing flies to 9 hours (ZT12-21) of heat at 29°C, a

temperature at which TrpA1 opens to drive depolarization of c584-Gal4 labeled cells. **A)**

Sleep rebound after thermogenetic sleep deprivation. Sleep rebound is calculated as the

difference between sleep time during the 15-hour recovery period from ZT21-ZT12 and

the equivalent 15-hour period during the baseline day. **B)** Sleep loss during

thermogenetic sleep deprivation. Sleep loss is calculated as the difference between sleep

time from ZT12-ZT21 on the baseline day and ZT12-ZT21 during 9 hours of

thermogenetic SD. **C)** Baseline daytime (DT) and nighttime (NT) sleep time. **D)** Sleep

graphs from a representative experiment with sleep on the baseline day, sleep on the

sleep deprivation day, and sleep on the recovery day superimposed for each genotype.

Bar graphs in A), B), and C) are pooled data from three independent experiments with

n=60-64 for each genotype in each experiment.

Figure 3-2: MI00393/+ Phenotype Does Not Map to Transposon Insertion
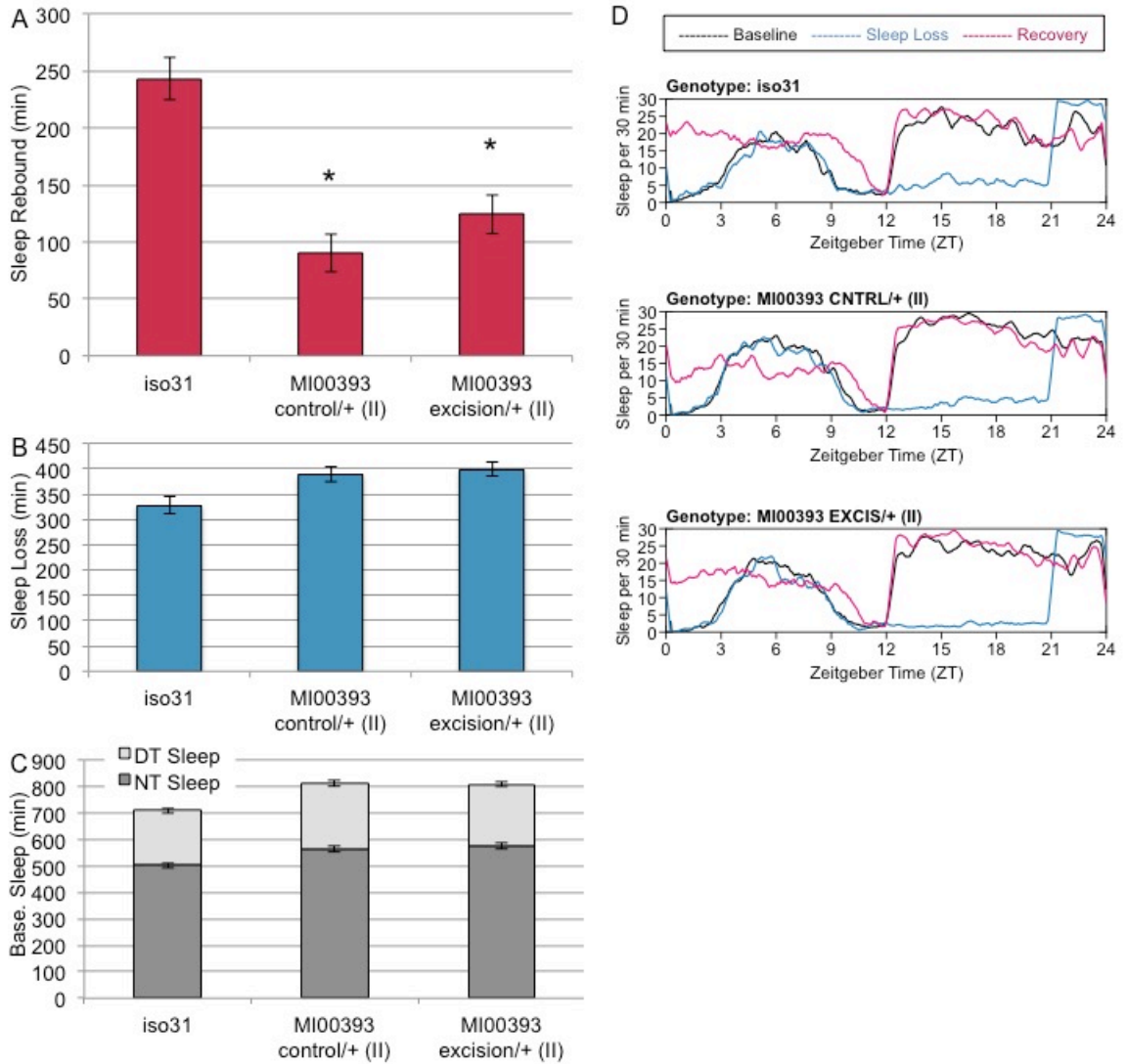
Figure 3-2: MI00393/+ Phenotype Does Not Map to Transposon Insertion

Thermogenetic sleep deprivation experiment with a precise excision of the MiMIC insertion in MI00393, compared to a control. Both the precise excision and control lines have an isolated MI00393 second chromosome in an otherwise iso31 background. All genotypes include c584-Gal4/+, UAS-TrpA1/+. Baseline data is collected at 21°C and sleep deprivation is produced by exposing flies to 9 hours (ZT12-21) of heat at 29°C, a temperature at which TrpA1 opens to drive depolarization of c584-Gal4 labeled cells. **A)** Sleep rebound after thermogenetic sleep deprivation. Sleep rebound is calculated as the difference between sleep time during the 15-hour recovery period from ZT21-ZT12 and the equivalent 15-hour period during the baseline day. **B)** Sleep loss during thermogenetic sleep deprivation. Sleep loss is calculated as the difference between sleep time from ZT12-ZT21 on the baseline day and ZT12-ZT21 during 9 hours of thermogenetic SD. **C)** Baseline daytime (DT) and nighttime (NT) sleep time. **D)** Sleep graphs from a representative experiment with sleep on the baseline day, sleep on the sleep deprivation day, and sleep on the recovery day superimposed for each genotype. Bar graphs in A), B), and C) are pooled data from two independent experiments with n=30-32 for each genotype in each experiment.

Figure 3-3: Comparison of Gene Expression Changes with SD in Wild-Type iso31 and MI00393/+ (II) Flies
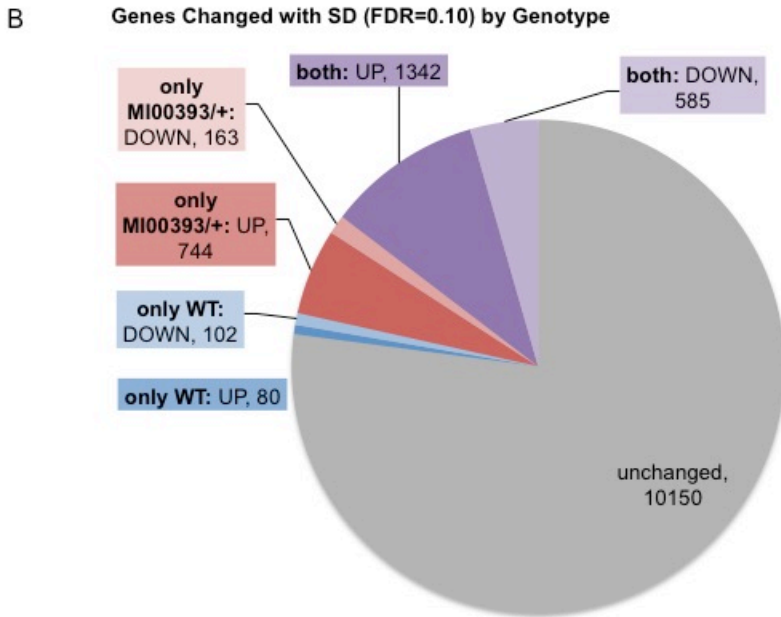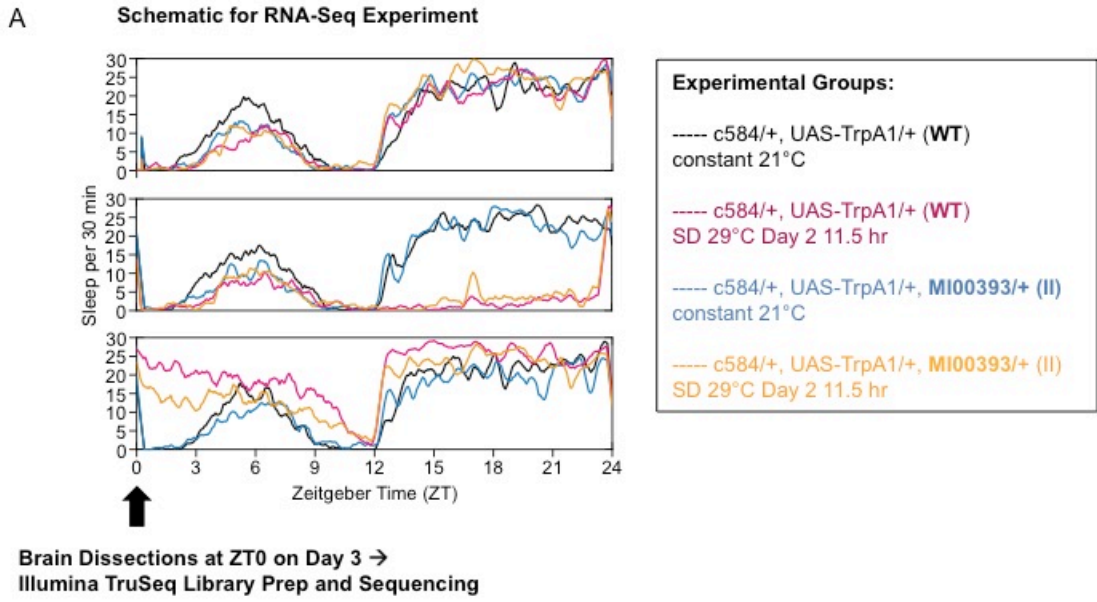
Figure 3-3: Comparison of Gene Expression Changes with SD in Wild-Type iso31 and MI00393/+ (II) Flies

A) Schematic for RNA-Seq Experiment. WT (c584/+, UAS-TrpA1/+) and MI00393/+ (c584/+, UAS-TrpA1/+, MI00393/+ made with the MI00393 chromosome II isolated line) flies were either kept at a constant 21°C or exposed to 11.5 hours of heat at 29°C from ZT12 to ZT23.5 to induce thermogenetic sleep deprivation. After the sleep deprivation period, all flies were removed from incubators at ZT0, and 20 brains per condition were dissected and collected for RNA extraction. This procedure was repeated on four separate days to create four independent sets of samples. Libraries were then prepared for Illumina sequencing. B) Numbers of genes changed with SD (FDR=0.10) in WT, MI00393/+ (II) flies, or both genotypes.

Figure 3-4: Baseline Gene Expression Differences Between iso31 and MI00393/+ (II) Suggest Reduced Wake-Promoting Stimulus in MI00393/+ (II)
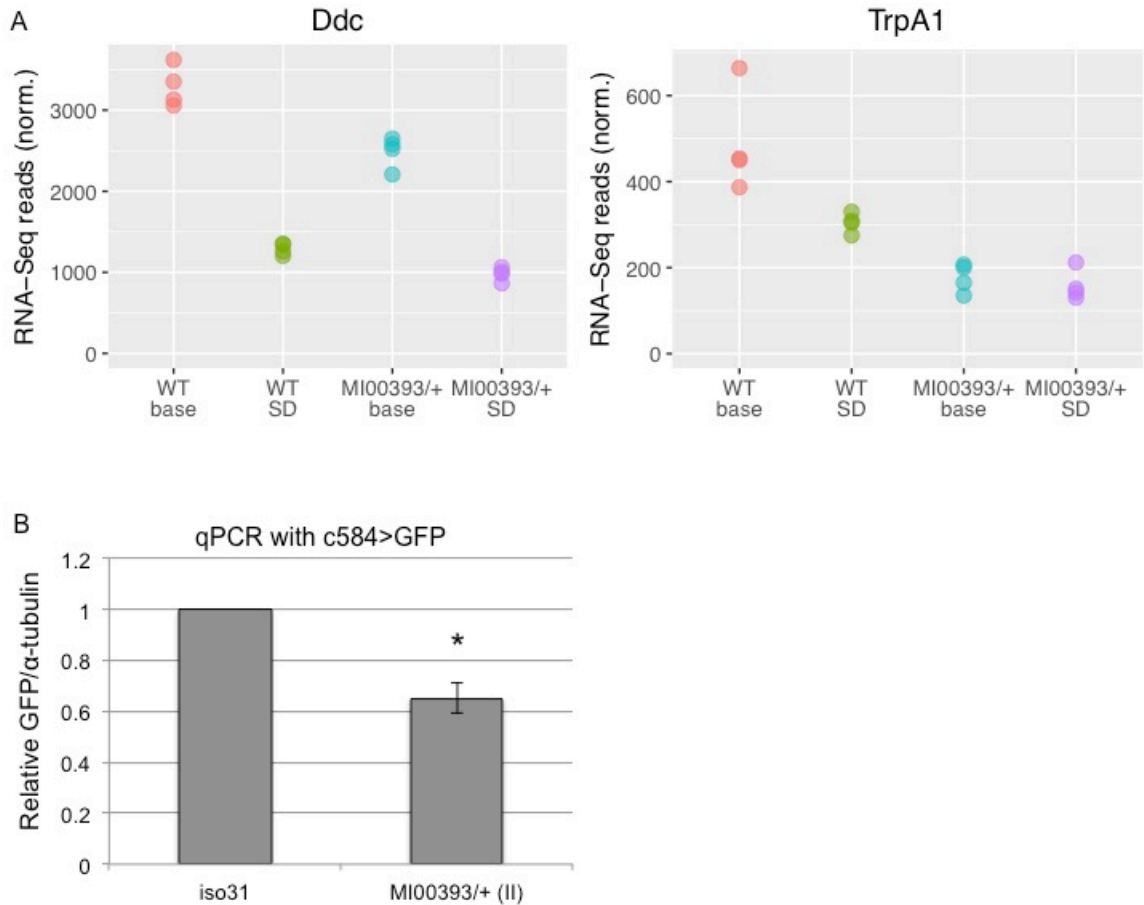
A) Normalized RNA-Seq data for *Ddc* and *TrpA1* reveal a difference in expression levels between iso31 and MI00393/+ (II) at baseline and further changes that occur with thermogenetic SD. Both differences are consistent with reduced wake-promoting stimulus in MI00393/+ (II) flies. **B)** GFP qPCR in c584-Gal4, UAS-GFP/+ flies reveals reduced Gal4-driven expression in MI00393/+ (II) flies compared to iso31. Both genotypes contain c584-Gal4, UAS-nGFP/+. Data from three independent experiments.

Figure 3-5: *Ddc* Mutations Partially Phenocopy MI00393/+ (II)
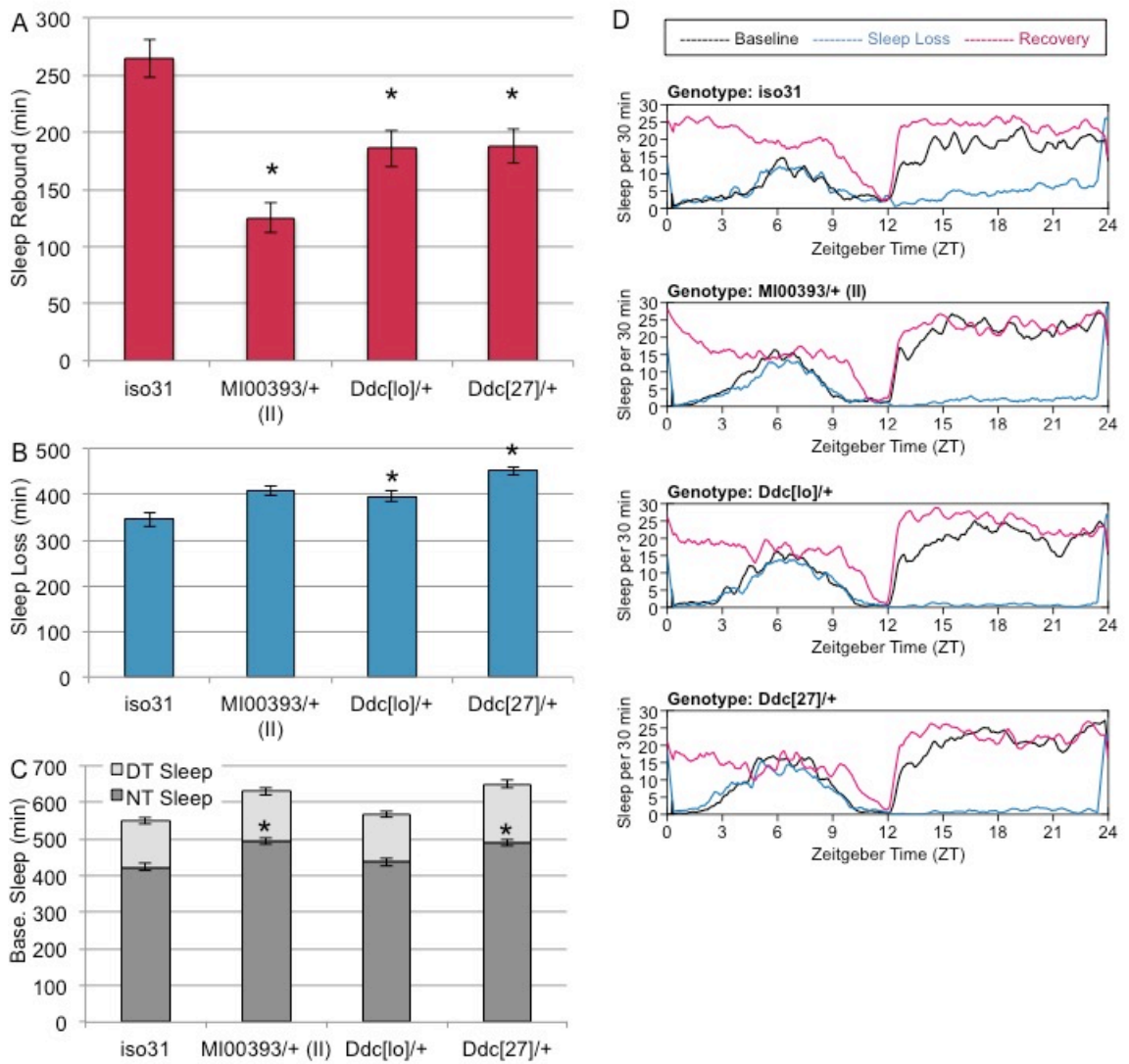
Figure 3-5: *Ddc* Mutations Partially Phenocopy MI00393/+ (II)

Thermogenetic sleep deprivation experiment with heterozygous *Ddc* alleles backcrossed into the iso31 wild-type background. All genotypes include c584-Gal4/+, UAS-TrpA1/+. Baseline data is collected at 21°C and sleep deprivation is produced by exposing flies to 11.5 hours (ZT12-23.5) of heat at 29°C, a temperature at which TrpA1 opens to drive depolarization of c584-Gal4 labeled cells. **A)** Sleep rebound after thermogenetic sleep deprivation. Sleep rebound is calculated as the difference between sleep time during the 12 hour recovery period from ZT0-ZT12 and the equivalent 12 hour period during the baseline day. **B)** Sleep loss during thermogenetic sleep deprivation. Sleep loss is calculated as the difference between sleep time from ZT12-ZT24 on the baseline day and ZT12-ZT24 during thermogenetic SD. **C)** Baseline daytime (DT) and nighttime (NT) sleep time. **D)** Sleep graphs from a representative experiment with sleep on the baseline day, sleep on the sleep deprivation day, and sleep on the recovery day superimposed for each genotype. Bar graphs in A), B), and C) are pooled data from three independent experiments with n=27-32 for each genotype in each experiment.

Figure 3-6: Additional UAS-construct Reduces TrpA1 Expression and Abolishes Sleep Rebound with a Smaller Effect On Sleep Loss
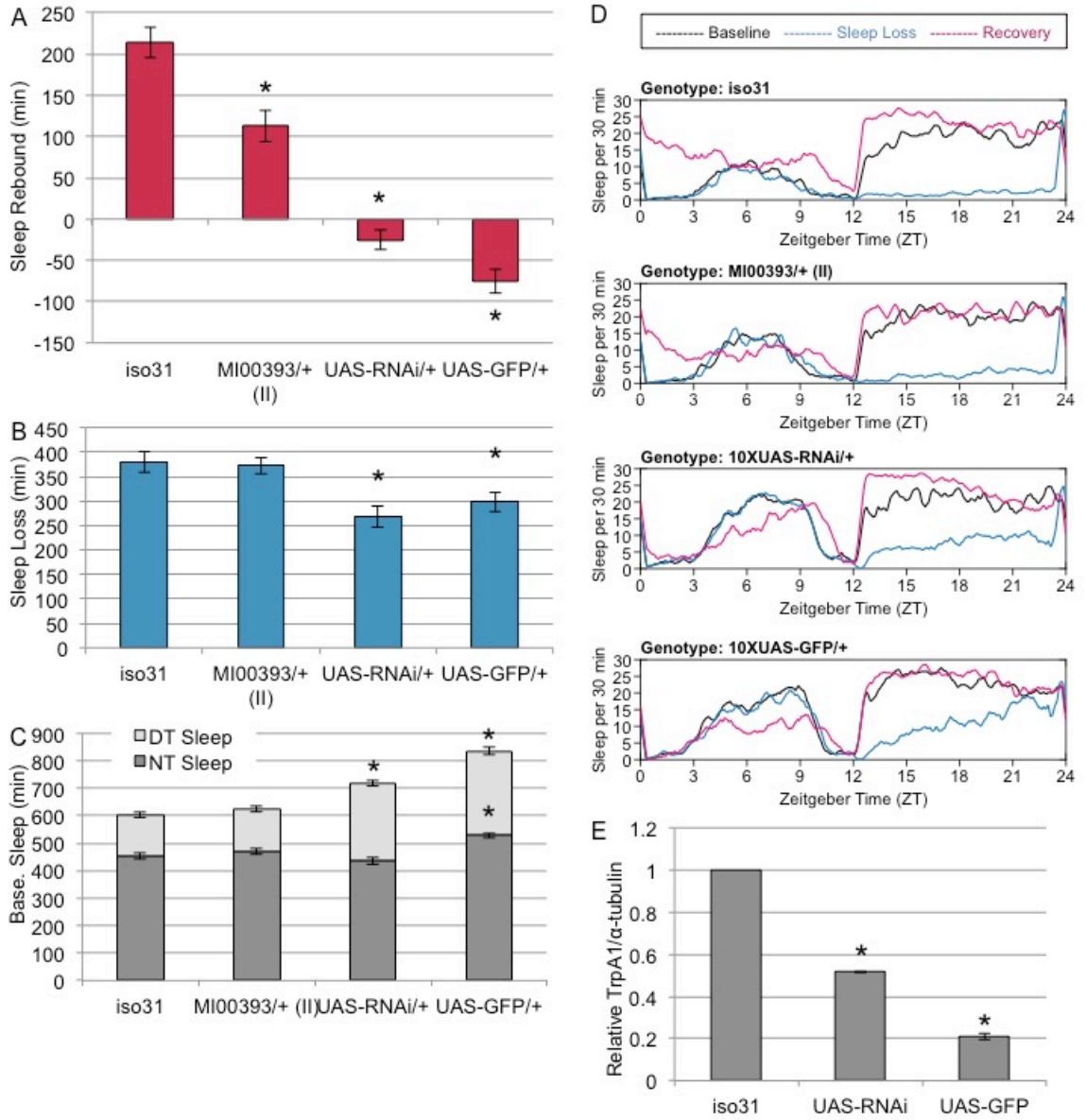
Figure 3-6: Additional UAS-construct Reduces TrpA1 Expression and Abolishes Sleep Rebound with a Smaller Effect On Sleep Loss

Thermogenetic sleep deprivation experiment in flies with 10X-UAS-RNAi or 10X-UAS-GFP constructs. All genotypes include c584-Gal4/+, UAS-TrpA1/+. Baseline data is collected at 21°C and sleep deprivation is produced by exposing flies to 11.5 hours (ZT12-23.5) of heat at 29°C, a temperature at which TrpA1 opens to drive depolarization of c584-Gal4 labeled cells. **A)** Sleep rebound after thermogenetic sleep deprivation. Sleep rebound is calculated as the difference between sleep time during the 12 hour recovery period from ZT0-ZT12 and the equivalent 12 hour period during the baseline day. **B)** Sleep loss during thermogenetic sleep deprivation. Sleep loss is calculated as the difference between sleep time from ZT12-ZT24 on the baseline day and ZT12-ZT24 during thermogenetic SD. **C)** Baseline daytime (DT) and nighttime (NT) sleep time. **D)** Sleep graphs from a representative experiment with sleep on the baseline day, sleep on the sleep deprivation day, and sleep on the recovery day superimposed for each genotype. Bar graphs in A), B), and C) are pooled data from two independent experiments with n=31-32 for each genotype in each experiment. **E)** *TrpA1* expression as measured by qPCR is reduced in flies with 10X-UAS-RNAi or 10X-UAS-GFP, suggesting Gal4 dilution. Data shown are from two independent qPCR experiments.

Table 3-1: Top 100 Genes (Lowest q-value) Changed with SD in WT Flies

Symbol	WT Control vs. SD q-value	WT Control vs. SD Fold Change	WT Control vs. SD Direction	MI00393 Control vs. SD q-value	MI00303 Control vs. SD Fold Change	MI00393 Control vs. SD Direction	Avg. # of Reads per Sample
Ddc	0	2.548	down	0	2.541	down	2012
CG31760	0	1.389	down	0.033	1.287	down	1536
CG1358	0	1.532	down	0	1.446	down	1354
CG4577	0	1.442	up	0.009	1.202	up	5391
CG44247	0	1.364	up	0	1.293	up	2211
CG14864	0	2.585	down	0.049	2.394	down	31
CG6511	0	4.545	up	0	3.676	up	336
CG14186	0	1.741	up	0.016	1.472	up	628
Cul1	0	1.458	up	0.008	1.446	up	1587
FoxP	0	1.784	up	0.012	1.525	up	2110
CG43102	0	1.403	up	0	1.294	up	2514
tud	0	1.219	up	0.076	1.139	up	3533
rgn	0	1.235	down	0.031	1.232	down	881
dally	0	1.514	down	0	1.4	down	1811
CG10254	0	1.284	up	0.001	1.27	up	671
gfzf	0	1.88	up	0.001	1.919	up	277
Xrp1	0	1.365	up	0.059	1.313	up	1550
Pitslre	0	1.544	down	0.009	1.55	down	3378
YT521-B	0	1.285	up	0.027	1.299	up	2032
Lk6	0	1.525	up	0	1.449	up	7673
CG1316	0	1.427	up	0	1.368	up	1214

Cul3	0	1.162	up	0.265	1.044	up	783
Ars2	0	1.323	up	0.016	1.239	up	1089
CG32756	0	2.482	up	0.016	2.226	up	256
CG14299	0	1.331	up	0.028	1.318	up	596
Ten-m	0	1.35	down	0.031	1.374	down	2957
CG42235	0	1.581	down	0.033	1.245	down	722
CG12858	0	1.465	down	0	1.425	down	2672
CG42575	0	1.171	up	0.015	1.241	up	4130
pdm3	0	1.374	down	0	1.428	down	446
Eaat1	0	1.551	down	0.045	1.345	down	3267
shi	0	1.281	down	0	1.269	down	7150
vap	0	1.387	up	0	1.356	up	965
CG1416	0	2.178	up	0.001	2.547	up	519
Cirl	0	1.244	down	0.044	1.253	down	3946
Atpalpha	0	1.472	down	0	1.456	down	27477
Ntf-2	0	1.785	up	0	1.926	up	460
pds5	0	1.219	up	0.07	1.041	up	563
Gad1	0	1.266	down	0	1.225	down	5845
pUf68	0	1.428	down	0.007	1.482	down	2566
CG15765	0	1.334	down	0.019	1.265	down	4602
CG11407	0	1.67	down	0.298	1.084	down	398
hrg	0	1.22	up	0.042	1.194	up	1518
ytr	0	1.582	up	0.035	1.52	up	941
CG14408	0	1.316	up	0.008	1.421	up	1062
EloA	0	1.379	up	0.016	1.311	up	372

Caper	0	1.712	down	0	1.488	down	859
pAbp	0	1.286	up	0.053	1.227	up	4572
CG14619	0	1.35	up	0	1.237	up	1831
inaE	0	1.545	down	0.03	1.437	down	785
hig	0	1.307	down	0.017	1.225	down	5179
Tsp5D	0	1.868	down	0.072	1.397	down	507
eRF1	0	4.157	up	0	3.592	up	1189
CG32000	0	1.58	up	0.064	1.329	up	11784
Sox102F	0	1.644	down	0.01	1.665	down	636
ClC-b	0	1.589	up	0	1.579	up	533
nrv2	0	1.699	down	0.074	1.327	down	5433
CG9153	0	1.818	up	0	1.862	up	2514
Pa1	0	1.616	up	0.052	1.782	up	192
Tsp42Ek	0	3.504	up	0.013	3.77	up	118
stau	0	1.319	up	0.043	1.219	up	1591
CG2269	0	1.071	up	0.154	1.082	up	7972
Aps	0	1.52	up	0.017	1.405	up	1294
Ef1alpha100E	0	1.515	up	0.007	1.54	up	4929
Droj2	0	1.594	up	0	1.816	up	1651
CG2918	0	1.395	up	0.029	1.459	up	1022
CG6424	0	1.185	up	0.047	1.172	up	8742
MICAL-like	0	1.69	up	0.026	1.526	up	660
HmgZ	0	3.754	down	0	3.833	down	615
CR45683	0	2.546	up	0	2.253	up	190
CG17490	0	2.241	up	0.03	2.032	up	422

CG5337	0	1.486	up	0.008	1.531	up	624
cwo	0	1.508	up	0.069	1.237	up	299
CG32164	0	1.585	up	0.019	1.459	up	198
ATP8B	0	1.413	up	0.069	1.283	up	999
CycG	0	1.439	up	0.037	1.306	up	3928
ab	0	1.433	down	0.038	1.646	down	380
elav	0	1.67	up	0.049	1.45	up	4187
CG5872	0	2.408	up	0	2.354	up	344
Hsp23	0	7.746	up	0	6.975	up	236
Dark	0	1.439	up	0.074	1.28	up	741
mthl8	0	1.751	up	0.003	1.502	up	2124
CG8216	0	1.498	down	0.265	1.128	down	247
pps	0	2.176	up	0.026	2.309	up	1203
lr76a	0	2.427	up	0.004	2.21	up	289
CR44662	0	3.585	up	0	2.923	up	75
tho2	0	1.342	up	0.04	1.248	up	733
Hsp68	0	7.376	up	0.013	5.821	up	80
Hsromega	0	5.014	up	0.031	4.478	up	3955
Ugt35b	0	1.527	down	0.257	1.114	down	1767
CG43191	0	43.866	up	0.004	66.545	up	88
CG31776	0	13.612	up	0.017	9	up	40
CG5618	0	6.023	up	0	7.882	up	309
Uhg5	0.001	2.048	up	0.035	1.916	up	455
Hop	0.001	2.593	up	0	2.646	up	322
CG31646	0.001	1.419	down	0	1.35	down	290

Hrb98DE	0.001	1.273	up	0	1.343	up	1908
CG33230	0.001	2.458	up	0.003	2.543	up	89
stj	0.001	1.422	down	0.023	1.395	down	2597
CR45479	0.001	3.32	up	0.006	2.625	up	67

Table 3-2: DAVID Term Clusters for Genes Changed with Thermogenetic SD in WT Flies (FDR=0.10)

Color Code for % of Genes Down- or Up-Regulated in Table 3-1 and Table 3-2
Down   Up

90%	80%	70%	60%	50%	50%	60%	70%	80%	90%

Enrich-ment Score	# of Genes	Genes Up	Genes Down	Cluster GO terms
3.57	245	168	77	**Nucleotide/nucleoside binding:** nucleotide binding, purine ribonucleotide binding, ribonucleotide binding, purine nucleotide binding, adenyl ribonucleotide binding, ATP binding, nucleoside binding, purine nucleoside binding, adenyl nucleotide binding
3.40	22	14	8	**Splicing:** regulation of alternative nuclear mRNA splicing, via spliceosome; regulation of RNA splicing; regulation of mRNA processing; regulation of nuclear mRNA splicing, via spliceosome
2.92	113	100	13	**Translation:** amino acid activation; ncRNA metabolic process; tRNA aminoacylation; tRNA aminoacylation for protein translation; ligase activity, forming carbon-oxygen bonds; ligase activity, forming aminoacyl-tRNA and related compounds; aminoacyl-tRNA ligase activity; tRNA metabolic process; translation
2.54	98	63	35	**Vesicle-mediated transport:** vesicle-mediated transport; membrane invagination; endocytosis; membrane organization; phagocytosis, engulfment; phagocytosis
2.18	46	38	8	**Ubiquitin metabolism:** ubiquitin protein ligase binding; cullin-RING ubiquitin ligase complex; enzyme binding; ubiquitin ligase complex; nuclear ubiquitin ligase complex; ubiquitin-dependent protein catabolic process
2.16	149	71	78	**Synaptic function:** synapse; postsynaptic membrane; glutamate receptor activity; cell junction; synapse part; extracellular ligand-gated ion channel activity; ligand-gated channel activity; ligand-gated ion channel activity; passive transmembrane transporter activity; channel activity; ion channel activity; substrate specific channel activity; extracellular-glutamate-gated ion channel activity; ionotropic glutamate receptor activity; gated channel activity; ion transport; cation channel activity; metal ion transmembrane transporter activity
2.05	136	108	28	**Macromolecule catabolism:** modification-dependent protein catabolic process; modification-dependent macromolecule catabolic process; small conjugating

				protein ligase activity; ubiquitin-protein ligase activity; cellular protein catabolic process; proteolysis involved in cellular protein catabolic process; protein catabolic process; acid-amino acid ligase activity; cellular macromolecule catabolic process; ligase activity, forming carbon-nitrogen bonds; ubiquitin-dependent protein catabolic process; macromolecule catabolic process; proteolysis
2.05	85	70	15	**RNA binding:** RNA binding; mRNA binding
1.72	97	50	47	**Neurotransmission:** synaptic transmission; cell-cell signaling; transmission of nerve impulse; secretion; secretion by cell; regulation of neurotransmitter levels; synaptic vesicle endocytosis; synaptic vesicle transport; neurotransmitter secretion; generation of a signal involved in cell-cell signaling; neurotransmitter transport; exocytosis; synaptic vesicle exocytosis; neurological system process
1.58	306	205	101	**Ion binding:** zinc ion binding; metal ion binding; transition metal ion binding; ion binding; cation binding
1.48	45	21	24	**Memory, olfaction, and cognition:** memory; learning or memory; learning; olfactory learning; olfactory behavior; chemosensory behavior; cognition
1.45	40	29	11	**Microtubule cytoskeleton and centrosome organization:** centrosome cycle; centrosome organization; microtubule organizing center organization; centrosome duplication; centriole replication; spindle organization; microtubule cytoskeleton organization
1.45	35	33	2	**Protein folding:** cis-trans isomerase activity; protein folding; peptidyl-prolyl cis-trans isomerase activity; regulation of cellular protein metabolic process
1.44	37	32	5	**Translation:** regulation of translation; posttranscriptional regulation of gene expression
1.44	13	4	9	**Regulation of neuronal system process:** regulation of system process; regulation of synaptic transmission; regulation of transmission of nerve impulse; regulation of neurological system process
1.41	57	43	14	**Cellular Stress:** cellular response to stress; response to DNA damage stimulus; DNA repair; DNA metabolic process
1.35	37	24	13	**Response to Heat and Environment:** response to temperature stimulus; response to heat; response to abiotic stimulus
1.33	5	4	1	**Regulation of Ion Transport:** regulation of ion transport; regulation of metal ion transport; regulation of calcium ion

				transport
1.33	54	40	14	**GTP/GDP binding:** GTP binding; guanyl ribonucleotide binding; guanyl nucleotide binding; GTPase activity; small GTPase mediated signal transduction
1.31	35	13	22	**Behavior:** adult behavior; locomotory behavior; adult locomotory behavior

Table 3-3: Top 100 Genes (Lowest q-value) Changed with SD in WT Flies but not (q>0.40) in MI00393/+ (II) flies

Symbol	WT Control vs. SD q-value	WT Control vs. SD Fold Change	WT Control vs. SD Direction	Avg. # of Reads per Sample	MI00393 Control vs. SD q-value	MI00393 Control vs. SD Fold Change	MI00393 Control vs. SD Direction
Gs2	0.005	1.425	down	3491	1	1.052	down
CG43707	0.005	1.293	down	2427	1	1.004	down
pkaap	0.007	1.439	up	374	1	1.031	up
CalpA	0.01	1.137	up	820	1	1.019	up
mnb	0.01	1.218	up	397	0.265	1.034	down
CG30105	0.011	2.068	up	11	1	1	unchanged
gek	0.013	1.053	up	779	1	1.025	down
CG7365	0.013	2.4	up	1	1	2.5	up
Fbw5	0.014	1.325	up	86	1	1.005	down
Acf1	0.015	1.205	up	513	1	1.008	up
CG7442	0.015	1.666	down	42	1	1.032	up
CG4409	0.016	1.417	down	559	1	1.008	down
CG9005	0.017	1.102	down	828	1	1.005	down
Zir	0.019	1.279	up	1756	1	1.035	up
Strica	0.019	4.545	up	5	1	1.384	up
CG11619	0.019	1.595	up	76	1	1.068	up
Unc-115b	0.019	1.62	up	69	1	1.015	down
CG8301	0.02	1.201	down	302	1	1.013	up
Axs	0.021	1.509	down	32	1	1	unchanged
l(2)35Bc	0.021	1.775	up	30	1	1.042	up
CR43459	0.021	1.317	up	57	1	1.073	up
Dad	0.022	1.123	up	232	0.062	1.082	down
CG7509	0.022	1.701	down	236	1	1.108	down
CG14688	0.022	1.655	down	144	0.283	1.106	up
CG17556	0.022	1.389	up	83	1	1.071	up
CG12994	0.022	1.425	down	88	1	1.058	up
pico	0.022	1.16	up	540	1	1.046	up

Orco	0.024	2.56	down	11	1	1.187	down
CG17018	0.024	2.587	up	98	0.119	1.087	down
l(2)03659	0.027	1.545	up	6	1	1.033	up
E23	0.028	1.368	down	89	1	1.017	down
Vha68-1	0.028	1.169	down	2437	1	1.022	up
CG9313	0.029	2.25	up	6	1	1.13	down
CG15728	0.031	2.8	down	4	1	1.058	up
serp	0.031	3.25	up	2	1	1.545	down
CG12926	0.032	1.354	down	163	1	1.049	down
CG8858	0.032	1.232	up	299	1	1.047	up
jnj	0.032	1.17	up	285	1	1.01	down
ninaB	0.035	1.427	down	324	1	1.055	down
Vha55	0.035	1.109	down	2291	1	1.01	down
Nmdar1	0.035	1.232	down	597	1	1.01	down
DIP1	0.035	1.15	down	2241	1	1.041	down
VhaPPA1-1	0.035	1.158	down	566	1	1.018	down
Rh50	0.035	1.337	down	651	0.283	1.069	up
EndoB	0.035	1.099	down	626	1	1.011	down
metro	0.035	1.196	down	566	1	1.019	up
AGO1	0.035	1.232	up	4956	1	1.022	up
Obp44a	0.036	1.895	down	427	1	1.014	up
CR44472	0.036	2.044	up	16	1	1.047	up
CR45054	0.037	1.844	up	24	1	1	unchanged
CR45161	0.037	1.378	down	52	1	1.032	up
CG14451	0.039	2	down	2	1	1.222	down
CG17387	0.039	2.375	down	2	1	1.083	down
CG18754	0.039	2.625	up	2	1	1.333	up
nAChRalpha2	0.04	1.162	down	446	1	1.027	down
CG9935	0.04	1.47	up	112	1	1.025	up
tefu	0.04	1.196	up	275	1	1.007	down
CR43957	0.04	1.569	down	26	1	1.103	down
Rop	0.043	1.384	down	1466	1	1.042	down

Ank	0.043	1.215	up	6524	1	1.029	up
DIP2	0.043	1.16	up	1908	1	1.021	up
CG45050	0.043	1.264	up	2975	1	1.009	up
mei-41	0.045	1.395	up	63	1	1.066	up
CG7191	0.045	1.335	up	119	1	1.039	up
CG7737	0.045	1.277	down	120	1	1.012	down
CG4835	0.045	3	up	1	1	2.5	up
CG7979	0.045	1.167	up	139	1	1.042	up
CG31467	0.045	1.544	up	25	1	1.049	up
hd	0.045	1.64	down	7	1	1.074	up
Fcp3C	0.048	2.1	down	3	1	1.083	up
Ir20a	0.048	2.2	up	1	1	1	unchanged
CG9294	0.048	2.2	down	1	0.145	1.428	up
slgA	0.049	1.077	down	3254	1	1.01	down
CG7420	0.049	1.432	down	27	1	1.096	up
CG7433	0.049	1.196	down	3553	1	1.023	down
DNApol-iota	0.049	1.133	down	209	1	1.046	down
CR44024	0.049	1.273	up	1462	1	1.003	down
CG7084	0.05	1.395	down	440	1	1.061	down
CG31475	0.052	1.27	down	206	1	1.014	up
hk	0.053	1.126	down	733	1	1.02	up
Ptp69D	0.053	1.171	up	706	1	1.005	up
List	0.053	1.235	down	469	1	1.027	up
Tusp	0.053	1.16	down	679	1	1.018	down
CG34126	0.053	1.068	down	1247	1	1.018	down
slim	0.053	1.113	up	763	1	1.022	up
CG10555	0.055	1.229	up	258	1	1.05	up
CG8818	0.055	1.411	down	238	1	1.041	up
Cyp12b2	0.055	1.204	down	284	1	1.009	down
CG5728	0.055	1.273	up	206	1	1.024	up
Tango5	0.055	1.19	down	285	1	1.003	up
CG34133	0.055	1.109	down	402	1	1.025	down

NKAIN	0.055	1.043	up	1052	1	1	up
CG15209	0.059	1.499	down	1222	1	1.061	down
CDase	0.059	1.353	down	1036	1	1.016	down
wech	0.059	1.215	up	1108	1	1.008	down
CG14044	0.06	1.307	down	15	1	1.234	down
CG3437	0.06	1.115	up	49	1	1.064	down
CG12590	0.06	1.95	down	6	1	1.24	down
CG17777	0.06	2	down	9	1	1.205	down
CG31898	0.06	1.88	down	22	1	1.032	down

Table 3-4: Top 100 Genes (Lowest q-value) Changed with SD in MI00393/+ (II) Flies but not (q>0.40) in WT Flies

Symbol	MI00393 Control vs. SD q-value	MI00393 Control vs. SD Fold Change	MI00393 Control vs. SD Direction	Avg. # of Reads per Sample	WT Control vs. SD q-value	WT Control vs. SD Fold Change	WT Control vs. SD Direction
CG2662	0.001	1.532	up	24	1	1.019	up
CG3353	0.003	1.92	up	45	1	1.427	up
CG15143	0.006	2.363	down	4	1	1.411	down
ACXC	0.006	2.75	down	15	1	1.131	down
CG31370	0.006	3.034	up	13	1	1.5	up
pirk	0.008	2.507	up	28	1	1.171	up
CG4089	0.009	1.523	up	60	1	1.091	up
CG10979	0.01	1.119	up	237	1	1.034	up
CG9422	0.012	1.503	up	78	1	1.009	up
CG5681	0.013	3.833	down	3	1	1.076	up
Rpl12	0.013	2.227	up	8	1	1.088	down
CG10916	0.017	1.518	up	86	1	1.148	up
sun	0.018	1.624	up	142	1	1.032	up
CG15892	0.018	3.833	up	7	1	1.064	up
CG13001	0.018	1.334	up	120	1	1.138	up
CG11454	0.018	1.405	up	68	1	1.092	down
MED4	0.018	1.727	up	23	1	1.18	up
Ubc4	0.019	1.339	up	225	1	1.145	up
Asciz	0.019	1.467	up	74	1	1.175	up
Scox	0.019	1.948	up	99	1	1.311	up
CG43861	0.02	1.22	down	139	1	1.044	down
thoc7	0.021	1.228	up	112	1	1.134	up
dyn-p25	0.021	1.594	up	43	1	1.116	up
CG9164	0.022	1.123	up	322	1	1.012	up
CG2685	0.023	1.469	up	62	1	1.202	up
CG3253	0.023	1.3	up	141	1	1.04	down

Hmg-2	0.024	1.939	up	27	1	1.049	down
CstF-64	0.024	1.775	up	30	1	1.238	up
CG10324	0.024	1.96	up	82	1	1.162	up
Prx6005	0.025	2.52	up	22	1	1.216	up
CG10463	0.025	1.613	up	37	1	1.356	up
CG4538	0.025	1.242	up	534	1	1.079	up
mus81	0.025	2	up	17	1	1.176	up
CG33267	0.025	1.354	up	25	1	1.357	up
CG3638	0.025	1.185	down	1071	1	1.039	down
coro	0.025	1.217	up	159	1	1.128	up
Marf	0.026	1.167	up	1417	1	1.04	up
CG11885	0.027	1.54	up	25	1	1.265	up
Gmd	0.027	1.476	up	72	1	1.12	up
CG11906	0.027	1.268	up	82	1	1.076	up
CG5567	0.027	1.343	up	146	1	1.179	up
Vps2	0.027	1.385	up	102	1	1.073	up
CG30371	0.027	2.6	down	1	1	1.2	down
nmd	0.028	1.363	up	221	1	1.135	up
l(1)G0230	0.028	1.356	up	283	1	1.009	down
HP5	0.028	1.192	up	261	1	1.085	up
CG8239	0.028	1.957	up	38	1	1.292	up
CG6878	0.028	1.455	up	57	1	1.158	up
MED19	0.028	1.406	up	152	1	1.136	up
Sod	0.029	1.872	up	226	1	1.172	up
mRpL49	0.029	2.008	up	45	1	1.188	up
tkv	0.03	1.277	up	580	1	1.07	up
smg	0.03	1.132	up	432	1	1.049	up
fend	0.03	1.358	down	336	1	1.113	down
CG6406	0.03	1.256	up	179	1	1.091	down
CG9801	0.03	1.241	up	311	1	1.074	up
Trx-2	0.03	1.361	up	242	1	1.006	up
CR45533	0.03	1.317	down	295	1	1.155	down

Prosalpha5	0.031	1.728	up	83	1	1.09	up
CG3621	0.031	1.613	up	37	1	1.279	up
Mst85C	0.031	1.38	up	68	1	1.163	up
CG18420	0.031	1.866	down	4	1	1.866	down
CG12125	0.031	1.418	up	175	1	1.085	up
dik	0.031	1.391	up	119	1	1.078	up
insv	0.031	1.696	up	36	1	1.072	down
CG3077	0.031	1.359	up	118	1	1.124	up
CG11986	0.031	1.453	up	33	1	1.035	up
CG14894	0.031	1.474	up	88	1	1.034	up
CG5934	0.031	1.362	up	58	1	1.288	down
Nhe2	0.031	1.12	down	2752	1	1.038	down
CR13130	0.031	1.532	up	19	1	1.025	down
Prosalpha4	0.032	1.577	up	80	1	1.153	up
CG3735	0.032	1.376	up	76	1	1.265	up
CG14982	0.032	1.251	up	524	1	1.046	up
CG44009	0.032	1.418	up	23	1	1.137	up
Scsalpha	0.033	1.372	up	356	1	1.06	up
Scp1	0.033	2.461	down	8	1	1.12	down
CG14971	0.033	1.37	up	204	1	1.193	up
CG10053	0.033	1.447	up	22	1	1.154	up
Miro	0.033	1.228	up	457	1	1.089	up
CG30340	0.033	2.666	down	1	1	1.333	down
CoRest	0.033	1.219	up	334	1	1.078	up
Fic	0.033	1.407	up	83	1	1.127	up
e(y)2	0.034	1.7	up	15	1	1.109	down
TfIIS	0.034	1.385	up	145	1	1.017	up
Uch-L5	0.034	1.421	up	72	1	1.154	up
MED15	0.034	1.456	up	167	1	1.199	up
mRpL24	0.034	1.515	up	171	1	1.19	up
CG8204	0.034	1.942	up	24	1	1.106	up
CG9804	0.034	1.716	up	36	1	1.389	up

MtnA	0.035	1.766	up	1351	1	1.146	up
CG10376	0.035	1.204	up	309	1	1.096	up
Drl-2	0.035	1.285	down	574	1	1.085	down
CG10209	0.035	1.256	up	97	1	1.062	up
ste24a	0.035	1.288	up	269	1	1.151	up
CG10469	0.035	1.33	up	34	1	1.1	up
Mtr3	0.035	1.734	up	32	1	1.327	up
CG11722	0.035	1.448	up	46	1	1.083	up
vig2	0.035	1.563	up	181	1	1.166	up

Table 3-5: DAVID Term Clusters for Genes Changed with Thermogenetic SD in MI00393/+ (II) Flies Only (FDR=0.10)

Enrich-ment	# of Genes	Genes Up	Genes Down	Cluster terms
2.71	64	61	3	**Organelle lumen:** mitochondrial lumen, mitochondrial matrix, intracellular organelle lumen, organelle lumen, membrane-enclosed lumen, nucleoplasm, nucleoplasm part, nuclear lumen
2.40	105	103	2	**Mitochondrion:** mitochondrion, mitochondrial part, mitochondrial envelope, organelle envelope, envelope, mitochondrial membrane, organelle membrane, mitochondrial inner membrane, hydrogen ion transmembrane transporter activity, cellular respiration, monovalent inorganic cation transmembrane transporter activity, organelle inner membrane, respiratory chain, respiratory chain complex IV, mitochondrial respiratory chain complex IV, generation of precursor metabolites and energy, electron transport chain, energy derivation by oxidation of organic compounds, oxidative phosphorylation, mitochondrial ATP synthesis coupled electron transport, ATP synthesis coupled electron transport, mitochondrial respiratory chain, oxidoreductase activity, acting on heme group of donors, oxidoreductase activity, acting on heme group of donors, oxygen as acceptor, cytochrome-c oxidase activity, heme-copper terminal oxidase activity, respiratory electron transport chain, mitochondrial electron transport, cytochrome c to oxygen, mitochondrial membrane part
2.35	17	16	1	**Cofactor/coenzyme metabolic process:** cofactor metabolic process, coenzyme metabolic process, cofactor biosynthetic process, coenzyme biosynthetic process
2.24	71	67	4	**Mitochondrial ribosomal protein, mitochondrial matrix, translation:** organellar large ribosomal subunit, mitochondrial large ribosomal subunit, mitochondrial lumen, mitochondrial matrix, organellar ribosome, mitochondrial ribosome, large ribosomal subunit, ribonucleoprotein complex, ribosomal subunit, structural constituent of ribosome, ribosome, translation, structural molecule activity
1.79	14	13	1	**Lipid binding:** phospholipid binding, phosphoinositide binding, lipid binding
1.75	5	4	1	**Vitamin biosynthetic process:** water-soluble vitamin biosynthetic process, water-soluble vitamin metabolic process, vitamin biosynthetic process, vitamin metabolic process
1.55	32	31	1	**Endomembrane system, RNA localization:** establishment of RNA localization, mRNA transport, RNA

				transport, nucleic acid transport, mRNA export from nucleus, RNA localization, RNA export from nucleus, nucleobase, nucleoside, nucleotide and nucleic acid transport, nuclear export, pore complex, nuclear pore, nuclear transport, nucleocytoplasmic transport, endomembrane system, nuclear envelope

Table 3-6: Gene Expression Differences Between iso31 and MI00393/+ Flies Under Baseline Conditions (FDR=0.40)

Gene Symbol	q-val	Fold Change	Avg. # of Reads per Sample
bowl	0	30.374	644
CG17018	0	11.253	98
CG18853	0	38.25	21
CG17684	0.055	3.997	237
Gadd45	0.069	2.694	80
CG33296	0.069	2.789	628
loh	0.071	1.746	79
CG32581	0.071	122.25	72
Cyp6a20	0.087	7.246	71
Lap1	0.099	1.493	658
Ady43A	0.111	2.501	160
CG11319	0.122	1.409	2702
CG43707	0.122	1.702	2427
Tsp42Ee	0.138	1.177	979
CG17167	0.235	1.77	36
CG40470	0.242	1.374	1931
CG15431	0.29	1.305	787
Ddc	0.317	1.321	2012
CG31760	0.319	1.229	1536
Mical	0.399	1.774	3014

Supplemental Figure 3-1: Principal Component Analysis of RNA-Seq Data
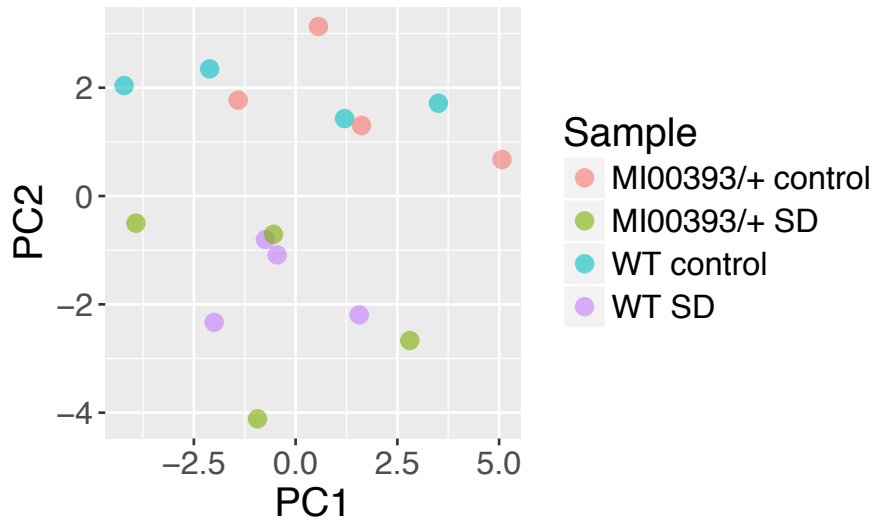
Principal component analysis reveals good separation of control and sleep deprivation (SD) groups on principal component two (PC2) but no apparent separation between genotypes.

Methods

Fly Stocks and Husbandry

Flies are maintained on standard cornmeal/molasses food. c584-Gal4, UAS-TrpA1 and MI00393 flies were described in Chapter 2. Other stocks were obtained from Bloomington Stock Center (BSC; Bloomington, Indiana), the Drosophila Genomics and Genetic Resources department (DGGR; Kyoto, Japan), or made as described. Stocks for mapping the MI00393 phenotype to a single chromosome were created in a series of crosses using iso31; Sco/CyO (BSC# 5907) iso31;; TM2/TM6cSb (BSC# 5906) and iso31; Sco/CyO; TM2/TM6bTb (Sehgal Lab) stocks. The MI00393 "excision" and "control" stocks were created by crossing the MI00393 stock to yw; Sco/SM6a-hsILMiT (BSC# 36311), subjecting progeny to a heat shock during gametogenesis to induce Minos transposase expression, then using single male progeny in subsequent crosses to re-isolate the MI00393 chromosome II (with or without the MiMIC transposon excised) in an otherwise iso31 background, as was done in initial chromosome mapping. In addition to the excision stock, this allowed the creation of a control stock subjected to the same exposure to transposase and series of crosses but with the MiMIC transposon retained.

Fly stocks carrying Ddc^{lo} (DGGR# 102105) and Ddc^{27} (BSC# 3109) alleles and TRiP RNAi construct JF02356 (BSC# 27030) were obtained from Bloomington Stock Center. Ddc^{lo} and Ddc^{27} were backcrossed into the wild-type iso31 background for 5 generations before testing behavior. Although relative levels of Ddc activity had been characterized

for these alleles previously (Wright *et al.* 1982), the causal mutations in *Ddc* had not been identified, so we used a series of PCR reactions to amplify the *Ddc* coding sequence in these stocks and submitted the PCR products to the University of Pennsylvania DNA Sequencing Core (Philadelphia, PA) for Sanger Sequencing. This revealed an A>G change in the coding sequence of *Ddc^lo* (genome position 2L:19117596) that results in a Lysine>Glutamate change in the *Ddc* polypeptide. We could not identify a coding change in *Ddc^27*; however we were able to identify a change in a Broad-Complex binding site previously implicated in epidermal expression of *Ddc* (Hodgetts *et al.* 1995; Chen *et al.* 2002) and a 12-base pair insertion in the *Ddc* 5' UTR. Further work will be needed to determine if either of these non-coding mutations is the causal change this allele. Importantly, the *Ddc^27* allele retained homozygous lethality even after backcrossing, suggesting that the null mutation is still present in this stock.

Crosses for behavioral and most molecular assays were set by crossing c584-Gal4, UAS-TrpA1 iso31 females to males of the test genotype, resulting in heterozygous progeny. In the experiment where GFP expression driven by c584-Gal4 was assessed by quantitative PCR, females of a c584-Gal4, UAS-nGFP iso31 genotype were crossed with males of the test genotypes.

Thermogenetic Sleep Deprivation and Sleep Behavior Assays

For thermogenetic sleep deprivation experiments, flies were raised at 19-20°C to 6-14 days old, loaded into locomotor tubes, and transferred to a 21°C 12 hour:12 hour light:dark cycle for habituation and collection of baseline data. After 4-5 days of habituation, flies were subjected to a high temperature of 29°C for 9 or 11.5 hours starting at ZT12. Behavioral data before, during, and after sleep deprivation were collecting using the *Drosophila* Activity Monitoring System (Trikinetics, Waltham, MA) and processed using pySolo (Gilestro and Cirelli 2009). Sleep rebound is calculated as the difference between sleep duration during the 15-hour (ZT21-12) or 12-hour (ZT0-12) recovery period and the equivalent baseline period. Sleep loss is calculated by subtracting sleep through the 9-hour or 12-hour sleep loss period from the equivalent baseline period.

Illumina RNA Sequencing

Flies were raised and loaded into DAM tubes and subject to heat to induce 11.5-hour thermogenetic sleep deprivation as done for behavioral assays. At ZT0 following sleep deprivation, 20 flies per condition were briefly anesthetized with CO_2 then put on ice at. Brains were dissected in ice cold PBS within 40 minutes of anesthetization, disrupted in TRIzol (Thermo Fisher), then stored at -80°C. Procedure was repeated four times for four independent sets of samples. Individuals performing the dissections were randomized such that each individual did dissections for a different group on each of the four sample collection days to avoid variable dissection quality becoming a confounding factor. After all samples were collected, chloroform was used to extract RNA to aqueous phase, which

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was then further purified Quiagen RNasy Mini Kit. Libraries for sequencing were prepared from RNA using the Illumina TruSeq Stranded mRNA Sample Prep kit. Sample quality control was conducted at the Wistar Institute Genomics Core (Philadelphia, PA). 100 base pair paired-end sequencing was performed on an Illumina HiSeq 2000 by the Beijing Genomic Institute/Children's Hospital of Philadelphia High Throughput Sequencing Center (Bejing, China and Philadelphia, PA).

Bioinformatics

Alignment, normalization, and statistical analysis were done in collaboration with the Institute for Translational Medicine Bioinformatics Group at the University of Pennsylvania Perelman School of Medicine (Philadelphia, PA). Reads were aligned to the *Drosophila melanogaster* Release 6 genome using STAR version 2.3.0e and normalized by resampling using PORT v.0.7 (https://github.com/itmat/Normalization). Statistical comparison of gene expression across conditions was done using PADE version 0.2.1a1 (https://github.com/itmat/pade), which employs a permutation analysis to assign a q-value to each gene (Grant *et al.* 2005). Once a gene expression change meeting the FDR cut-off of 10% was identified in one genotype (q<0.10), a more lenient FDR of 40% was used to determine if the change also occurred in the second genotype (q<0.40). This approach improves our confidence in our "genotype only" gene lists and avoids the many false positives that would have occurred in these lists otherwise, with comparatively few false positives in the "both genotypes" list. DAVID analysis of enriched Gene Ontology (GO) terms was done by uploading lists of genes to DAVID 6.7

Web Service and applying Functional Annotation Clustering to group similar GO terms together. Principal components analysis was done using data from all genes with an average of 5 or more reads per sample using the prcomp function in R version 3.4.1.

Quantitative PCR

4-6 flies per group were briefly anesthetized and put on ice. Brains were dissected in ice cold PBS. RNA extraction was done with Qiagen Mini or Micro Plus RNeasy kits with on-column DNase digestion. Reverse strand cDNA synthesis was done with the Applied Biosystems High-Capacity cDNA Reverse Transcription kit and quantitative PCR was done with standard SYBR Green reagents on a Viia7 Real-Time PCR system (Thermo Fisher Scientific) using the Relative Standard Curve procedure. GFP primers were designed by hand (Forward: 5' GAAGGTGATGCAACATACGG 3', Reverse: 5' ACAAGTGTTGGCCATGGAAC 3'). TrpA1 primers primer sequences were obtained from GetPrime (http://bbcftools.epfl.ch/getprime) (Forward: 5' GAATGGCGACTTTAATGCG 3', Reverse: 5' CAATAGATAGTCCAGAGCGTC 3'). Expression was normalized to α-tubulin (Forward: 5' CGTCTGGACCACAAGTTCGA 3', Reverse: 5' CCTCCATACCCTCACCAACGT 3').

Statistics for Behavioral Assays

Statistics on behavioral data were calculated using R version 3.4.1. Statistical significance for sleep duration was calculated by ANOVA followed by Tukey's Honest

Statistical Difference post-hoc test. Differences in sleep duration were considered

statistically significant with a p<0.05 in both pooled data and in the majority of individual

experiments.

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Chapter 4: Conclusions and Future Directions

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In the past two decades, sleep research has seen a rapid explosion in the identification of genes, neurotransmitters, and neurons that influence sleep-wake regulation. These findings have also revealed specific mechanisms that allow sleep to change in the face of environmental, social, and metabolic conditions. The identification of "the" sleep homeostat – the force that increases sleep drive in response to long periods of wakefulness – is in some ways considered the elusive Holy Grail of basic sleep research. Indeed, our motivation for conducting a screen to identify genes essential for sleep rebound was to identify key components of such a homeostat. However, the work presented here and elsewhere suggests a shift in thinking may be necessary – rather than trying to identify "the" homeostat, we may need to think of sleep regulation as many forces that adjust sleep to match the environmental needs of an animal, in a way that flexibly balances the brain need for sleep with what is advantageous in the external conditions an animal finds itself in. Homeostasis is likely just one aspect of a more complex model for sleep regulation, and seems to be carried out by different mechanisms in different circumstances.

Research into sleep homeostasis has rested on several assumptions that come from early research on mammalian sleep. One assumption is that sleep regulation can be neatly split into a circadian process and a homeostatic process, which are independent from each other and which follow predictable kinetics under a variety of environmental scenarios and perturbations. Another assumption is that homeostatic sensors of sleep need are reading and responding to global molecular changes that likely reflect the function of sleep. These assumptions are not unfounded. Indeed, to the first point, mathematical models with a circadian component and a homeostatic component can elegantly describe

many, though not all, of the behavioral phenomena observed in sleep research (Borbely 1982; Daan *et al.* 1984; Franken *et al.* 2001; McCauley *et al.* 2009). The second point has been highly influenced by the finding that the cerebro-spinal fluid of animals that have been sleep deprived can induce sleep in non-deprived animals (Kubota 1989), leading some researchers to hypothesize the existence of global somnogens with potent sleep-inducing properties that are produced by neurons as they start to use up whatever resource sleep functions to restore (Porkka-Heiskanen and Kalinchuk 2011).

However, our growing understanding of the molecular biology of sleep has not supported the idea of a single homeostatic process or the idea of a key global somnogen. Many potential somnogens have been identified – neurochemicals that do indeed increase with sleep deprivation and have sleep inducing effects – but genetic studies have revealed the limited roles of these chemicals (Mizoguchi *et al.* 2001; Deboer *et al.* 2002; Stenberg *et al.* 2003; Huang *et al.* 2005; Bjorness *et al.* 2009). Each appears to influence only a subset of the phenomena that have traditionally been thought to be part of the homeostatic process, with relatively modest effects. The idea that changes in somnogens reflect global changes related to sleep function is also likely to be inaccurate. The somnogen adenosine, for example, was once thought to reflect brain-wide energy metabolism. Based on more recent work, however, it seems that adenosine is likely working through a specific circuit, where production relevant for sleep rebound is dependent on cholinergic cells in the basal forebrain (Porkka-Heiskanen and Kalinchuk 2011).

We add to this data our findings regarding sleep rebound. Like others, we find that sleep loss generated by different neural mechanisms produces different magnitudes of sleep rebound (Thimgan *et al.* 2010; Suzuki *et al.* 2013; Seidner *et al.* 2015; Machado *et al.* 2017) (Chapter 2, Figure 2-1). We see that different behaviors associated with sleep homeostasis are influenced by different genetic factors, and specifically find that mutations can have dramatic effects on sleep rebound with little to no effect on baseline sleep (Chapter 2, Figure 2-3 and Figure 2-S3). We also provide evidence that sleep rebound can be reduced by limiting the amount of wake-promoting stimulus used to keep flies awake, even when the extent of sleep loss is unaffected (Chapter 3, Figure 3-5 and Figure 3-6). This points to a mechanism of sleep rebound in which it is not sleep loss *per se* but the activity of wake-promoting neurons that produces increased sleep the following sleep deprivation. We propose a mechanism where homeostatic plasticity in sleep-regulatory circuits acts in opposition to wake-promoting stimuli to increase the propensity for sleep, resulting in sleep rebound when the wake-promoting stimulus stops or is overcome (Figure 4-1).

Below, we discuss experiments to identify homeostatic plasticity and underlying molecular mechanisms able to produce stable changes in a sleep-regulatory circuit following thermogenetic sleep deprivation (SD). We envision that homeostatic plasticity occurs in a circuit made up of the wake-promoting dopaminergic PPM3 neurons that we activate to produce thermogenetic SD and the sleep-promoting ExFl2 neurons in the dorsal fan-shaped body. ExFl2 are neurons thought to be downstream partners of PPM3s, and can be silenced by dopamine to produce wake (Ueno *et al.* 2012; Liu *et al.* 2012; Pimentel *et al.* 2016). Although this work focuses on a single circuit, there is the potential

for similar mechanisms to exist in any sleep-regulating circuit across the animal kingdom.

Homeostatic plasticity as a mechanism for sleep rebound

Homeostatic mechanisms exist throughout the brain to keep neuronal properties within acceptable parameters in the face of changing inputs (Marder and Goaillard 2006; Turrigiano 2012). Homeostatic plasticity must co-exist with Hebbian mechanisms that exist to re-enforce relevant signals (Turrigiano 2017). That homeostatic plasticity often affects a whole neuron, rather than a single synapse, and occurs on a longer time scale than Hebbian plasticity is likely what allows it to occur without erasing or interfering with information stored by Hebbian mechanisms.

Homeostatic plasticity has been well-characterized in several systems. In the *Drosophila* neuromuscular junction, a retrograde signal up- or down- scales neurotransmitter release from the pre-synaptic neuron when the neurotransmitter-evoked response in the post-synaptic muscle is inhibited or promoted (Frank 2014). In mammalian cortical neurons, each neuron has a firing-rate set point that it returns to after perturbations, primarily by cell-autonomously up- or down-scaling AMPA receptor expression, although presynaptic changes affecting excitatory and inhibitory inputs also occur (Turrigiano 2012). These phenomena act on time scales from minutes to hours long, and invoke many of the same molecules involved in other forms of neuronal plasticity. Although homeostatic plasticity has been studied in greatest detail in the systems mentioned above, homeostatic mechanisms exist throughout organisms and cell types. Indeed, in mammalian dopaminergic circuits, both pre- and post-synaptic homeostatic mechanisms have been

observed in response to both up- and down-regulation of dopamine signaling (Zigmond 1997; Jones *et al.* 1998; Bezard *et al.* 2003; Perez *et al.* 2008; Azdad *et al.* 2009; Bergstrom *et al.* 2011; Friedman *et al.* 2014; Fieblinger *et al.* 2014). Given the diversity of homeostatic mechanisms that exist to regulate neuronal activity throughout the brain, it is not unexpected that in the face of a long-term wake-promoting stimulus there might be some compensation in a sleep-wake regulatory circuit to favor sleep.

Sleep and synaptic homeostasis have been connected before, though in a different context. A well-known hypothesis known as the Synaptic Homeostasis hYpothesis or "SHY" proposes that a function of sleep is to allow a time for global homeostatic down-scaling of synapses that balances long-term potentiation taking place during wake (Tononi and Cirelli 2006). In investigating this hypothesis, numerous groups have shown that genes involved in synaptic plasticity or synaptic homeostasis are also involved in sleep-wake regulation: these include CREB, NFκB, *FMR1*, *homer*, *Alk*, *Nf1*, *Adar*, *cv-c* in *Drosophila* (Hendricks *et al.* 2001; Williams *et al.* 2007; Bushey *et al.* 2009; Naidoo *et al.* 2012; Donlea *et al.* 2014; Bai and Sehgal 2015) and CREB, BNDF, TNFα in mammals (Kushikata *et al.* 1999; Graves 2003; Krueger 2008; Faraguna *et al.* 2008; Bachmann *et al.* 2012; Watson *et al.* 2014). Note that there is significant overlap between genes involved in homeostatic and Hebbian plasticity. In many of these cases, it is not clear whether the observed effects on sleep are a result of global changes in synaptic strength, which would fit with SHY, or an effect in a specific circuit, which might suggest a mechanism similar to that discussed here.

In order to investigate the hypothesis that homeostatic plasticity in the neuronal circuit used to produce sleep loss drives subsequent rebound, techniques to interrogate neuron physiology are needed. Classical electrophysiology is one such method, however to do electrophysiology on PPM3 or ExFl2 cells, a large amount of brain matter above these cells needs to be removed, potentially severing relevant connections. Electrophysiology is also extremely low throughput, and cannot resolve issues that arise when heterogeneous cell groups such as PPM3s or ExFl2s are the targets of study. An alternative approach is optical electrophysiology – the use of fluorescent sensors that reflect neuronal activity (Lin and Schnitzer 2016).

Direct evidence of homeostatic plasticity as a mechanism that might underlie sleep rebound could be acquired by identifying a relevant physiological parameter in the sleep-regulating neuronal circuit, showing that the parameter changes after thermogenetic sleep deprivation, and then showing that the changes do not occur with sleep deprivation in mutant flies with reduced rebound. One relevant parameter might be the ExFl2 response to dopamine. Electrophysiology has been used to characterize the silencing of ExFl2 cells upon dopamine application, which is reflected by a decrease in input resistance and membrane time constant (Pimentel *et al.* 2016). Dopamine also provokes a large calcium influx in these cells (Nguyen 2017). Thus, a calcium sensor such as GCaMP6 (Chen *et al.* 2013) or a fast voltage sensor such as ASAP2f (Yang *et al.* 2016) could be used to characterize ExFl2 response to dopamine, construct a dose response curve, and then determine whether ExFl2 responses to dopamine are changed after thermogenetic sleep deprivation in a way that would favor sleep. Other relevant physiological parameters might be ExFl2 intrinsic activity, responses to other neurotransmitters, or anatomical

changes that reflect an increase or decrease in connectivity to other sleep-regulating areas.

Homeostatic plasticity might also work by affecting transmitter release in the neurons used to induce sleep loss. This type of homeostatic mechanism could be revealed by examining evoked PPM3 transmitter release, which could by produced by expressing P2X2, an ATP-sensitive sodium channel, in PPM3s and applying a low dose of ATP. An indirect way to detect a change in evoked transmitter release from PPM3s might be to measure the evoked physiological response in ExFl2 neurons in response to PPM3 stimulation. More direct approaches to identifying homeostatic mechanisms affecting PPM3s could involve quantifying evoked synaptic vesicle release using false fluorescent neurotransmitters (Gubernator *et al.* 2009) or synapto-pHlourin (Miesenbock *et al.* 1998). Both of these fluorescent tools are packaged into synaptic vesicles and produce a change in fluorescence upon vesicle exocytosis, allowing time course and magnitude of vesicle release following a stimulus to be measured. False fluorescent neurotransmitters are also packaged into vesicles through the same molecular mechanisms as ordinary catecholamines, so false fluorescent neurotransmitters can additionally act as a readout of vesicle loading. Finally, anatomic changes that suggest homeostatic plasticity might also be observed in this set of neurons.

Different roles for dopamine receptors in sleep loss and sleep rebound

Our work suggests a role for dopamine in both keeping flies awake and in producing subsequent sleep rebound. Dopamine is a potent wake-promoting stimulus in *Drosophila* and has been well-studied in the contexts of both sleep and memory. One interesting

aspect of dopamine signaling in *Drosophila* is apparently different roles of two DA1-like dopamine receptors, *Dop1R1* and *Dop1R2*, even within the same cells. Both of these receptors have been implicated in mediating the wake-promoting effects of dopamine, but in different ways. *Dop1R1* mutations prevent the wake-promoting effects of thermogenetically activating dopaminergic neurons or feeding flies L-DOPA, and these effects can be rescued by expressing *Dop1R1* in ExFl2 cells (Ueno *et al.* 2012; Liu *et al.* 2012). However, *Dop1R1* knockdown in ExFl2 cells did not have any effect on baseline sleep, while *Dop1R2* knockdown in ExFl2 neurons does increase baseline sleep and alters the sensitivity of these cells to applied dopamine (Ueno *et al.* 2012; Pimentel *et al.* 2016).

Dop1R1 and *Dop1R2* also have different effects on memory, although the details remain unclear. Whereas *Dop1R1* is required for olfactory memory acquisition (Kim *et al.* 2007) and likely transmits valence cues necessary for associative memory (Perisse *et al.* 2013), *Dop1R2* has a more complex role, and has been linked to forgetting, memory consolidation, and in shaping the interconnectivity of dopamine neurons in mushroom body circuits that gives rise to compartmentalization of dopamine signaling (Berry *et al.* 2012; Cohn *et al.* 2015; Plaçais *et al.* 2017). The role in forgetting in particular suggests that *Dop1R2* might in some way have effects that oppose the molecular changes produced by *Dop1R1*, at least on long time scales.

There also seem to be differences between the effects of *Dop1R1* and *Dop1R2* knockdown on the physiological properties of cells. Although both have homology to mammalian DA1 receptors, these receptors have different reported effects on ExFl2 physiology – *Dop1R1* is required for DA-induced increases in cAMP (Ueno *et al.* 2012),

while *Dop1R2* is required for DA-induced increases in calcium (Nguyen 2017). It also remains unclear which G protein signaling pathways are important for *Dop1R1* and *Dop1R2* signaling. That *Dop1R1* is necessary for a DA-induced increase in cAMP in ExFl2 cells suggests Gs coupling. Indeed, expressing the catalytically active subunit of PKA in ExFl2 cells results in decreased sleep, suggesting that cAMP-PKA signaling is capable of silencing the sleep-promoting ExFl2 cells (Liu *et al.* 2012). On the other hand, it has been suggested that *Dop1R2*-mediated silencing mechanisms act through Gi or Gq, because targeting these G proteins produces similar effects as *Dop1R2* knockdown on aspects of ExFl2 physiology (Pimentel *et al.* 2016; Nguyen 2017).

Although both *Dop1R1* and *Dop1R2* seem to facilitate the short-term wake-promoting effects of dopamine, it is possible that either or both of these receptors also have roles in producing a homeostatic response to increased dopaminergic signaling. Thus, an area of future research will determine the effects of *Dop1R1* and *Dop1R2* knockouts on both sleep loss produced by thermogenetic SD and subsequent rebound. Given its more complex role in memory and forgetting, *Dop1R2* seems the more likely candidate for mediating homeostatic compensation to increased DA signaling, while published data have demonstrated that *Dop1R1* is necessary for sleep loss induced by elevated dopamine. This result would provide a molecular basis for the apparent disconnect between sleep loss and sleep rebound observed in our data, and provide a starting point for further experiments to elucidate the mechanisms of homeostatic compensation in the circuit.

Genes that might be downstream mediators of homeostatic plasticity

In addition to dopamine receptors, we can identify other candidate genes that might play a role in homeostatic plasticity of this circuit. For any gene that might be part of the mechanism producing homeostatic plasticity in the PPM3>ExFl2 cell circuit, a role can be tested by knocking down the gene in the cells of interest using RNAi. In order to knock down genes in ExFl2 cells using the Gal4/UAS system while testing the response to thermogenetic sleep deprivation, an alternative thermogenetic method would be developed that uses the LexA/LexAOp binary expression system. A tyrosine hydroxylase (TH)-LexA construct that expresses in dopaminergic cells paired with LexAOp-TrpA1 could be one such method that would evoke sleep loss through a similar molecular mechanism as our current thermogenetic approach.

As mentioned previously, several genes with known roles in synaptic plasticity or function have already been implicated in *Drosophila* sleep behavior – these include the Rho-GAP *cv-c*, which has been implicated specifically in homeostatic plasticity at the Drosophila NMJ (Pilgram *et al.* 2011); *homer*, implicated in homeostatic plasticity at hippocampal neurons in mammals (Sala *et al.* 2005); as well as genes such as NFκB, *Alk*, *FMR1*, *Adar*, and *insomniac*, which have roles in synaptic strength and function even if they have not been linked specifically to homeostatic plasticity (Zhang *et al.* 2001; Heckscher *et al.* 2007; Rohrbough *et al.* 2012; Maldonado *et al.* 2013; Li *et al.* 2017).

Of these, *cv-c* and *insomniac* are particularly compelling because their effects on sleep have already been linked to the circuit we are interested in. *cv-c*, which in the NMJ is required postsynaptically to downregulate presynaptic transmitter release (Pilgram *et al.*

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2011), is a sleep-promoting molecule with function that maps to the ExFl2 neurons (Donlea *et al.* 2014). In a scenario where persistent activation of dopaminergic neurons leads to a down-regulation in dopamine release, *cv-c* might be a key non-cell-autonomous signaling molecule necessary for these changes.

Insomniac, a *Cullin-3* BTB adaptor, is a sleep-promoting molecule with function that seems to depend on dopamine, although specific cells where *insomniac* is required have not been identified (Stavropoulos and Young 2011; Pfeiffenberger and Allada 2012). *Insomniac* is localized to synapses, where it modulates synaptic function (Li *et al.* 2017). These findings are particularly interesting because *Cullin-3*, which physically interacts with *insomniac* and also regulates sleep, is one of few genes in our RNA-Seq data set with expression that is changed with sleep deprivation in wild-type flies but not in MI00393/+ mutant flies. Thus, these two genes might be mediators of homeostatic plasticity that occurs in wild-type flies to produce rebound but does not occur in MI00393/+ flies.

Although existing data provide some hints at mechanisms that might underlie homeostatic plasticity within this neural circuit, an unbiased approach could also be employed. We note that it is quite likely that molecular changes that reflect homeostatic plasticity would not be reflected in our RNA-Seq data, which is taken from whole brains and does not reflect any post-transcriptional mechanisms that might be at work. Translating Ribosome Affinity Purification is a next-generation sequencing approach that overcomes both of these shortcomings by allowing tagging of specific neurons and assessment of transcripts that are bound to ribosomes, indicating active translation

(Heiman *et al.* 2014). This approach could be used in either PPM3 or ExFl2 neurons to identify genes with changes in translation following sleep deprivation. The potential to identify novel plasticity mechanisms using an unbiased approach is particularly important given that homeostatic plasticity in *Drosophila* has been primarily studied at the NMJ; homeostatic plasticity in CNS circuits might occur by different mechanisms.
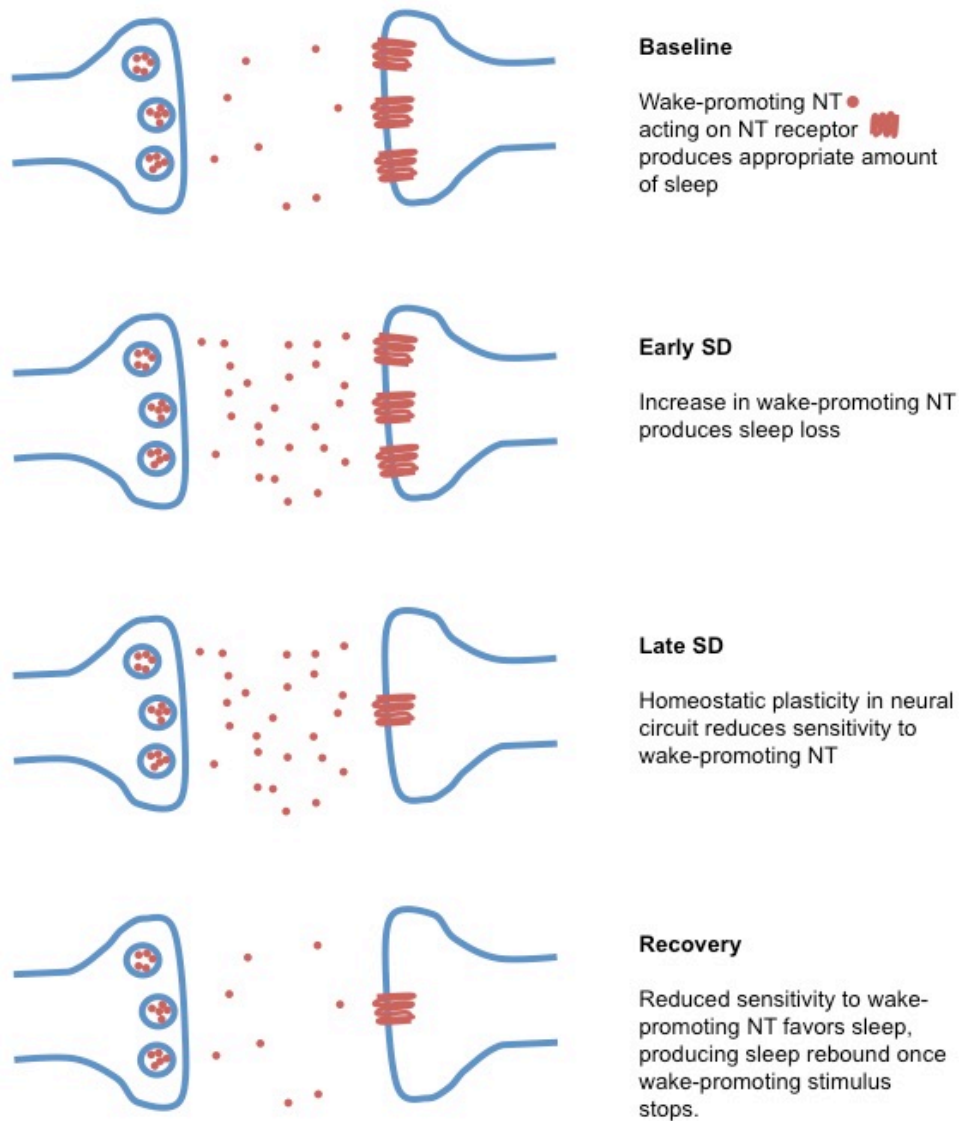
Finally, it is worth noting that although we envision baseline differences in *Ddc* expression and the different signaling roles of dopamine receptors as upstream steps that affect the initiation of homeostatic plasticity, changes in expression of these genes might also be effectors of a homeostatic mechanism. Indeed, decreases in *Ddc* and *Dop1R1* transcript levels are observed after thermogenetic sleep deprivation, although these changes occur in both wild-type and MI00393/+ flies, and therefore do not seem to be responsible for the differences in rebound sleep that we observe between these genotypes.

Concluding Remarks

The work presented here challenges many widely held assumptions about the nature of sleep homeostasis, and suggests homeostatic neuronal plasticity as a potential mechanism for the sleep rebound that occurs after a population of wake-promoting neurons is thermogenetically activated to induce sleep loss. Although we have not yet obtained direct evidence of homeostatic plasticity in this circuit, it would not be unexpected given the diversity of homeostatic plasticity mechanisms that exist. Future investigation will harness the power of genetically encoded sensors that reflect neuronal activity to determine the nature of homeostatic plasticity in this circuit, if it exists, and further genetic studies will be conducted to elucidate molecular mechanisms that underlie such

plasticity. Our work, in combination with previously published data, already suggests

some potential molecular mediators, although unbiased work may be successful in

identifying novel regulators of both sleep and homeostatic plasticity in the *Drosophila*

CNS.

Figure 4-1: Homeostatic Plasticity in Sleep-Regulating Circuits as a Potential Mechanism for Sleep Rebound

Baseline

Wake-promoting NT acting on NT receptor produces appropriate amount of sleep

Early SD

Increase in wake-promoting NT produces sleep loss

Late SD

Homeostatic plasticity in neural circuit reduces sensitivity to wake-promoting NT

Recovery

Reduced sensitivity to wake-promoting NT favors sleep, producing sleep rebound once wake-promoting stimulus stops.

Figure 4-1: Homeostatic Plasticity in Sleep-Regulating Circuits as a Potential Mechanism for Sleep Rebound

At baseline, normal amounts of the wake-promoting neurotransmitter (NT) act on the NT receptor to produce normal amounts of sleep. SD is produced by increasing wake-promoting NT release. Over time, excess wake-promoting NT triggers homeostatic plasticity in the circuit. This could manifest in a number of different ways, but is depicted here as a reduction in NT receptor levels. When SD stops, the reduced sensitivity to the wake-promoting NT results in higher sleep drive, producing sleep rebound. Not depicted: in mutants with reduced wake-promoting NT release during SD, less homeostatic plasticity occurs, resulting in less rebound.

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